MICROBIAL DEGRADATION OF LIGNIN SUBSTRUCTURE MODEL COMPOUNDS, *d,l*-SYRINGARESINOL AND RELATED DERIVATIVES

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CONTENTS :

PREFACE

Lignin, one of the principal source of the aromatic ring in nature, is a major constituent of cell walls in vascular plants, and a most abundant organic substance next to cellulose on earth. The microbial degradation of lignin holds an important part of the earth's carbon cycle. Lignin biodegradation is a slow process in natural environments, and lignin carbons are known to be incorporated into soil humus[1]. Woody plants are mainly degraded by the fungi, which are classified as white-rotter(Basidiomycetes and a few Ascomycetes), brown-rotter(Basidiomycetes) and soft-rotter(Fungi Imperfecti and Ascomycetes) based on the types of decay[2,3]. Of these, white-rot fungi are the most efficient lignin degraders in nature. Some bacteria(Nocardia, Streptmyces) and soil fungi(Fusaria, Aspergillus) are also known to degrade lignin partly[3].

The knowledge of the lignin biodegradation and transformation has greatly advanced during the past several years[3-5]. Moreover, interests in the application of bio-ligninolytic systems has been considerably increased; especially, attention has been given to bio-mechanical pulping and waste treatment[2,6]. However, elucidation of the chemistry and biochemistry of lignin biodegradation by fungi is rather a difficult task because of the structural complexity of lignin polymer and unique nature of its degradation. Lignin formation is initiated by peroxidase catalyzed oxidation of *p*-hydroxycinnamyl alcohols and followed by non-enzymic random coupling of the resulting radical species[7]. Consequently, lignin polymer has neither optical activity, crystallinity nor regularity in contrast to other natural polymers.

In these circumstances, studies with lignin substructure model compounds have considerably contributed to the understanding of the lignin

biodegradation. Lignin contains various linkages between phenylpropane units such as arylglycerol-β(α)-aryl ether[β(α)-*O*-4'], phenylcoumaran[β-5'], diarylpropane[β-1'], resinol[β-β'], biphenyl[5-5'], and diphenyl ether[4-*O*-5'][8]. The β-aryl ether dimer has often been used by many workers[3], because this substructure is most abundant in lignin.

In 1979, the present author started degradation studies using *d,l*-syringaresinol as a substrate and a soil fungus *Fusarium solani* M-13-1 as a degrader. At that time, degradation pathway for this substructure by fungi was not known and vanillic acid and vanillin had been reported as degradation products of *d,l*-pinoresinol by two white-rot fungi[9]. *d,l*-Syringaresinol, a β-β' linked dimer, is one of the important structural units in hardwood lignin. Occurrence of this substructure has been evidenced by its isolation as a hydrolysis product of hardwood[10] and bamboo[11] lignins. The frequency of β-β' linkage in lignin has been estimated to be 3% for birch lignin and 5% for beech lignin based on the acidolysis and thioacetolysis studies, respectively[12]. The corresponding guaiacyl compound, *d,l*-pinoresinol, has not been found in the acidolysis mixture of softwood or hardwood lignin. The ^{13}C-NMR spectrum of spruce wood lignin also indicated a very few content of this structure[13] : only two groups reported the occurrence of this unit in softwood lignin[14,15]. Hence, in the present investigation, *d,l*-syringaresinol was mainly used as a model representing the β-β' linked substructure in hardwood lignin.

First of all, an improved synthetic method for sinapyl alcohol was devised because *d,l*-syringaresinol is readily obtained by dehydrogenation of the alcohol with peroxidase and hydrogen peroxide system[16] (Chapter I).

In Chapter II, the results of degradation of *d,l*-syringaresinol by *F. solani* M-13-1 was discussed. The fungus was isolated from soil by an enrichment culture method using DHP(dehydrogenation polymer of coniferyl

alcohol, a synthetic lignin) as a sole carbon source[17]. Oxidation of the substrate by peroxidase/H_2O_2 was also examined and discussed in relation to the fungal degradation. The degradation studies with *F. solani* were extended using a trimeric lignin model compound composed of β-*O*-4' and syringaresinol substructures, the synthesis of which was described in Chapter I. It was expected that the use of higher oligolignols in biodegradation would provide missing links in the degradation process between dilignols and lignin.

In Chapter III, investigation on the degradation of *d,l*-syringaresinol and *d,l*-pinoresinol by a white-rot fungus *Phanerochaete chrysosporium* (= *Sporotrichum pulverulentum*) was described. *P. chrysosporium* is the most extensively studied white-rotter in this field[4,5]. Culture parameters affecting the ligninolytic activity in the fungus have been elucidated by Kirk and his co-workers[18-21]. Degradation studies with model compounds were conducted using the optimized ligninolytic cultures of this fungus[22]. Cellulose degradation by the fungus has been investigated by Eriksson's group[23].

Biphenyl linkage(5-5' linkage) is also one of the important substructures in lignin and the frequency has been estimated to be 10% for spruce lignin and 4.5% for birch lignin[12]. The relative resistance of this substructure to fungal attack has been demonstrated[24]. As an earlier study indicated[25], guaiacyl model compounds including *d,l*-pinoresinol are largely condensed to give biphenyl dimers as major products in fungal cultures producing phenol-oxidizing enzymes. The degradation of the biphenyl compound was presumed to be closely related to the aromatic ring cleavage, and therefore, degradation of biphenyl models, by *P. chrysosporium* was also investigated as described in Chapter III

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CHAPTER I

SYNTHESES OF *d,l*-SYRINGARESINOL AND GUAIACYLGLYCEROL-β-SYRINGARESINOL ETHER

INTRODUCTION

The useful synthetic method for 2,6-diaryl-3,7-dioxabicyclo[3,3,0]-octane type lignans has recently been reported by Fujimoto *et al.* with special interests in *d,l*-pinoresinol synthesis[1]. Nevertheless, dehydrogenation of sinapyl alcohol with peroxidase and hydrogen peroxide system is most convenient for preparation of *d,l*-syringaresinol ; this procedure gives the desired compound in about 90% yield[2]. However, usual synthetic method for sinapyl alcohol results in low yield[3]. Accordingly, an improved synthetic method for the alcohol was devised in connection with *d,l*-syringaresinol synthesis.

The general synthetic method for lignin substructure model compounds via β-hydroxy ester as a key intermediate has been developed by Nakatsubo[4]. By the method, Nakatsubo and his co-workers synthesized various dimeric and trimeric model compounds including arylglycerol-β-aryl ether, phenylcoumaran, diarylpropane and pinoresinol. In the present Chapter, a trimeric lignin model compound composed of β-*O*-4 and syringaresinol substructures was also synthesized by application of the method. The frequency of β-*O*-4 linkage in lignin substructures has been estimated to be about 50% for spruce and beech wood lignin[5]. Guaiacylglycerol-β-syringaresinol ether thus synthesized is considered to be a good model for hardwood lignin ; this trilignol has been isolated from Yachidamo wood as a hydrolysis product[6].

d,l-Syringaresinol synthesized was used in the degradation studies in Chapter II and III. Guaiacylglycerol-β-syringaresinol ether and its syringyl analog, syringylglycerol-β-syringaresinol ether, were used as substrates in Chapter II.

RESULTS AND DISCUSSION

Sinapyl alcohol has been synthesized by the method described by Freudenberg *et al.*[3] which involves the reduction of ethyl 4-*O*-acetyl sinapate to the alcohol in low yield(40 - 47%). Therefore, attention was focused to find a protecting group for the phenolic hydroxyl group which is stable for lithium aluminium hydride reduction and easily deprotected by subsequent treatment to give desired alcohol in good yield. As shown in Fig.1,

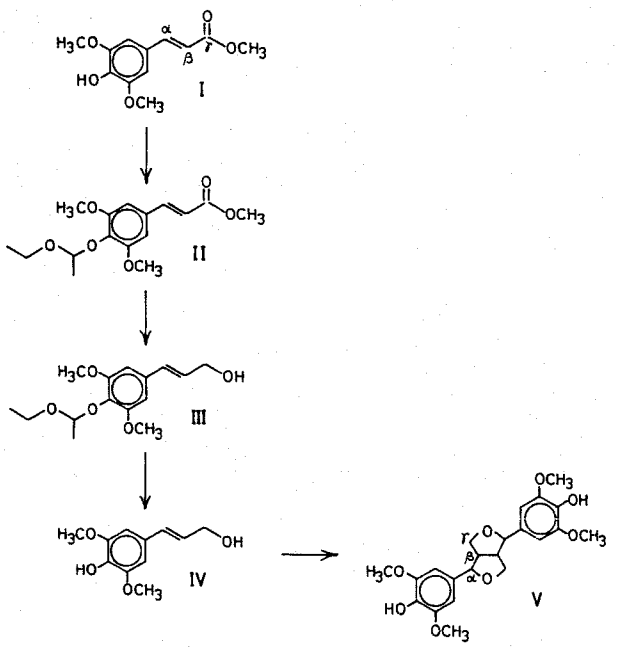

Fig. 1. Synthetic route for *d,l*-syringaresinol(V)

methyl sinapate(I) prepared by condensation of syringaldehyde and malonic

acid and subsequent esterification, was converted to its ethoxyethyl ether (II) by treatment with ethyl vinyl ether and D,L-camphorsulfonic acid for 15 - 20 min at 0°C in about 90% yield. Ethyl vinyl ether was selected as a reagent in consideration of the steric environment of the phenolic hydroxyl group : both *ortho*-positions are substituted with methoxyl groups. 2,3-Dihydropyran was found not to be suitable because of the steric hindrance. Reduction of II to the alcohol III with lithium aluminium hydride in anhydrous tetrahydrofuran(THF) at -35°C proceeded smoothly, although small amounts of impurities were detected by TLC analysis. The resulting alcohol was immediately subjected to mild hydrolysis in THF-water(9 : 1,v/v) mixture using pyridinium *p*-toluenesulfonate(PPTS) as a catalyst for 70 min at room temperature ; PPTS has been reported as a mild and efficient catalyst for the deprotection of tetrahydropyranyl ethers[7]. Crude sinapyl alcohol thus obtained was used directly to prepare *d,l*-syringaresinol(V) with peroxidase/H_2O_2. All steps of this procedure described here were conducted successively. Figure 2 shows ^{1}H-NMR spectrum of *d,l*-syringaresinol diacetate.

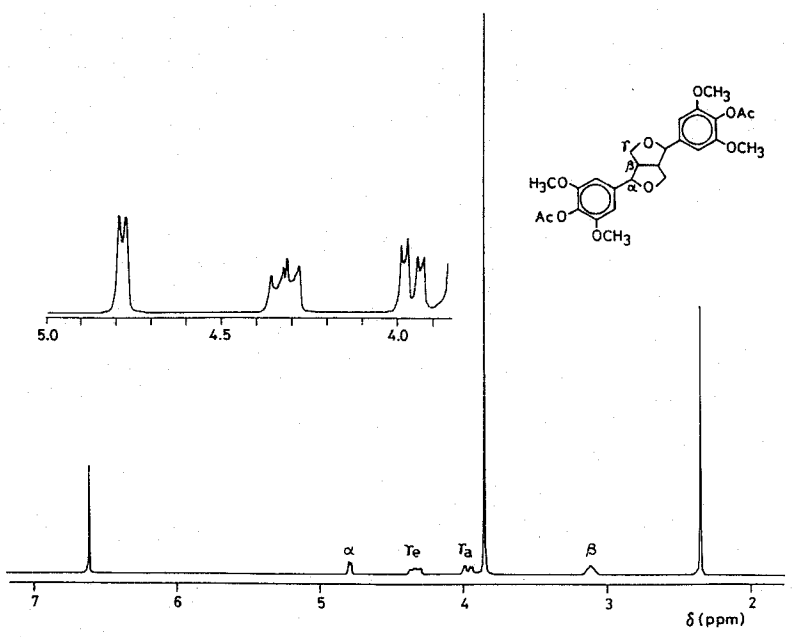

Fig. 2. ^{1}H-NMR spectrum of *d,l*-syringaresinol diacetate

In the present synthesis, sinapyl alcohol was not isolated in crystalline form, but this method was found to be suitable for *d,l*-syringaresinol synthesis ; overall yield of V from II was 66.5%. Some modifications[8] will be needed for preparation of pure sinapyl alcohol in high yield.

Synthetic route for guaiacylglycerol-β-syringaresinol ether(X) is shown in Fig. 3. Syringaresinol(V) was converted to its monobenzyl ether (VI) by treatment with benzyl chloride, KI and NaH in *N,N*-dimethylformamide (DMF) at 50°C, but the yield of the product was low(about 50%) because the two phenolic hydroxyl groups are synthetically equivalent and have the same reactivity. Attempts to devise an improved method are now in progress.

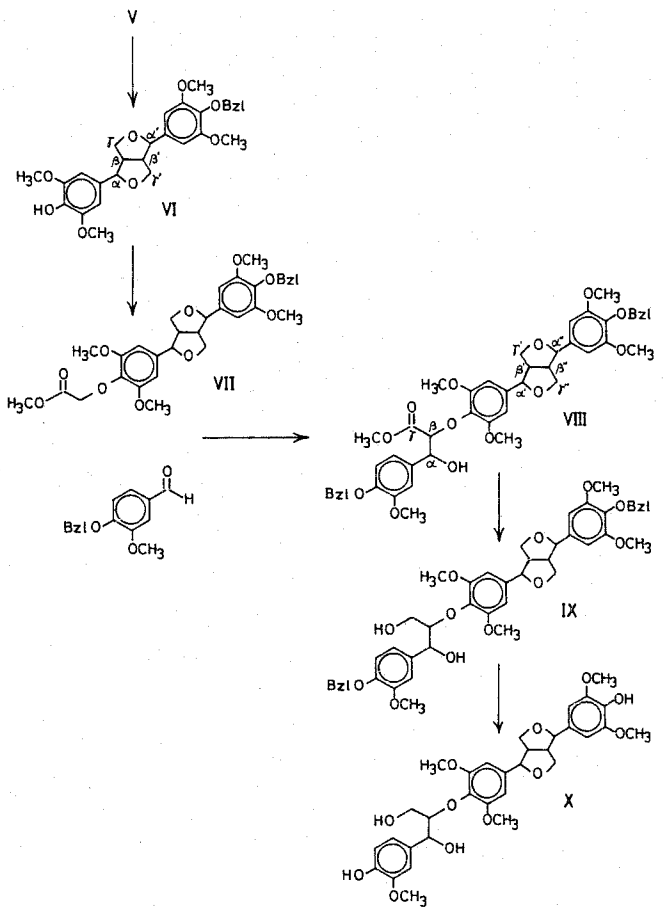

Fig. 3. Synthetic route for guaiacylglycerol-β-syringaresinol ether(X)

The monobenzyl ether(VI) was converted to the α-phenoxy acetate derivative (VII) by treatment with methyl monochloroacetate, KI and K_2CO_3 in acetone at reflux temperature in a high yield. Condensation of VII with benzyl vanillin[9] by the use of lithium diisopropylamide(LDA) in anhydrous THF below -72°C afforded the expected β-hydroxy ester(VIII) ; *erythro*(VIIIe) and *threo*(VIIIt) forms with a ratio of 9 : 1 in about 72% total yield. These isomers were separated by preparative TLC. The β-hydroxy ester derivative obtained by the condensation of ethyl 2-methoxyphenoxy acetate with benzyl vanillin in the presence of LDA has been found to be a mixture of *erythro/threo*(3 : 1) isomers[9]. The present result can be ascribed to the participation of the six-membered transition state which favors for the *erythro* form than the other under the influence of additional steric hindrance by the syringyl nucleus. In the present synthesis, the *erythro* product(VIIIe) was used for the subsequent steps. Reduction of VIII with lithium aluminium hydride in anhydrous THF at 50°C gave diol derivative(IX) in 85.4% yield. Finally, the desired trilignol(X) was obtained by catalytic hydrogenation on 10% palladium/carbon in methanol-dioxane(3 : 1) mixture with about 90% yield.

The mass spectrum of X did not show molecular ion peak(M^+), but the tetraacetate of X showed its M^+ at *m/z* 782 in the mass spectrum. The spectroscopic data, UV, IR, ^{1}H-NMR(acetate, 90 MHz) and MS(acetate) of X were in good agreement with those of the compound isolated as a hydrolysis product of Yachidamo wood[6]. The previously assigned structure was thus confirmed by the present synthesis.

Trilignol X as a substrate for degradation studies was synthesized without separation of the β-hydroxy ester intermediate. Figure 4 shows ^{1}H-NMR spectrum(200 MHz) of X(*erythro/threo* mixture) tetraacetate.

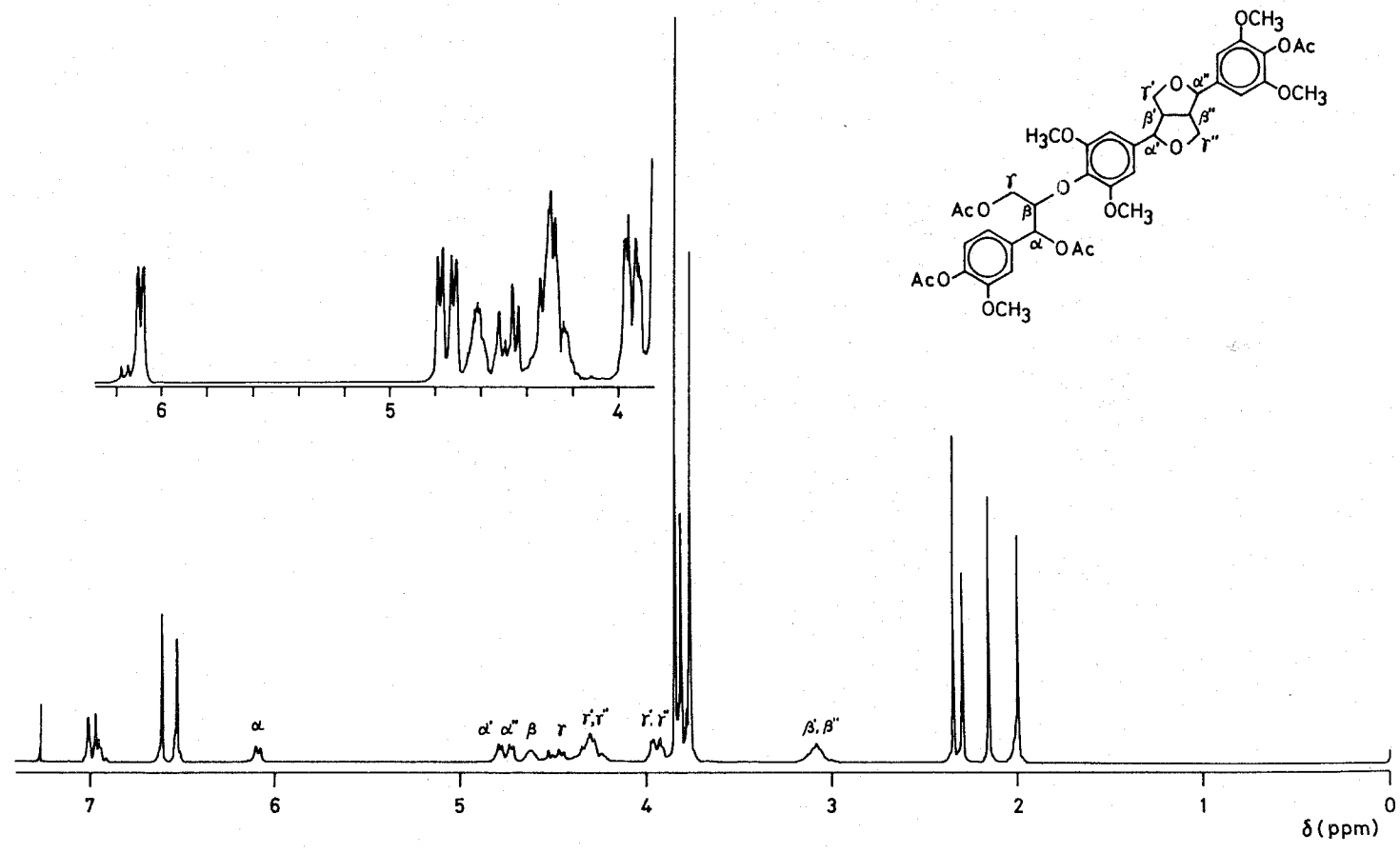

Fig. 4. ^{1}H-NMR spectrum of guaiacylglycerol-β-syringaresinol ether tetraacetate(*erythro/threo* mixture)

EXPERIMENTAL

All melting points were uncorrected. A Hitachi model 200-20 double beam spectrometer and a Jasco IR-S were used for UV and IR spectra, respectively. ^{1}H-NMR spectra(90 MHz) were taken by the use of a Hitachi R-22 high resolution NMR spectrometer using TMS as an internal standard. ^{1}H-NMR(200 MHz) and ^{13}C-NMR(50 MHz) spectra were measured with a Varian XL-200 NMR spectrometer. Chemical shifts and coupling constants(J) are given as δ values and Hz, respectively. Mass spectra were run on a Shimadzu-LKB 9000 gas chromatograph - mass spectrometer(70 eV). Preparative TLC was done with silica gel TLC plates coated with Kieselgel PF_{254}(Merck).

Methyl sinapate(I)

This compound was synthesized from syringaldehyde by condensation with malonic acid in dry pyridine in the presence of piperidine and anilin at 50°C, and subsequent esterification with methanol in the presence of *p*-toluenesulfonic acid and 2,2-dimethoxypropane[10] at reflux temperature.

Mp. 89.0 - 89.5°C. IR ν_{max}^{KBr} cm^{-1} : 3350, 2930, 1724, 1707, 1607, 1521 991. ^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 3.77(3H,s,γ-$COOCH_3$), 3.88(6H,s,Ar-OCH_3), 5.82(1H,s,Ar-OH), 6.22(1H,d,J=16 Hz, β-CH-), 6.68(2H,s,Ar-H), 7.51(1H,d,J= 16 Hz, α-CH-).

Methyl 4-O-(α-ethoxy)ethyl sinapate(II)

Ethyl vinyl ether(3.8 ml) and camphorsulfonic acid(150 mg) were added to a stirred solution of methyl sinapate(4.76 g, 20 mmol) in 30 ml of methylene chloride dried over alumina(Woelm B, Akt 1) at 0°C. After stirring for 15 min, 0.1 ml of triethylamine was added and the reaction mixture was partitioned between ethyl acetate and a saturated $NaHCO_3$ solution. The organic layer was washed with a saturated NaCl solution, dried over Na_2SO_4

and evaporated under reduced pressure to give a crystalline residue which was recrystallized from *n*-hexane and methylene chloride(5.47 g, 88.1%).

Mp. 89 - 90°C. IR ν_{max}^{KBr} cm^{-1} : 2830, 1726, 1638, 1585, 1033. ^{1}H-NMR (90 MHz, $CDCl_3$) δ(ppm) : 1.16(3H,t,J=7.2 Hz, $-OCH_2\underline{CH_3}$), 1.47(3H,d,J=5.3 Hz, $-CH\underline{CH_3}$), 3.77(3H,s,γ-$COOCH_3$), 3.84(6H,s,Ar-OCH_3), 3.40 - 4.16(2H,m,$-O\underline{CH_2}CH_3$), 5.26(1H,q,J=5.3 Hz, $-\underline{CH}CH_3$), 6.28(1H,d,J=16 Hz, β-CH-), 6.68(2H,s,Ar-H), 7.54 (1H,d,J=16 Hz, α-CH-).

4-O-(α-ethoxy)ethyl sinapyl alcohol(III)

To a stirred solution of $LiAlH_4$(758 mg, 20 mmol) suspended in 35 ml of anhydrous THF, 6.21 g(20 mmol) of compound II in 35 ml of anhydrous THF was added dropwise over a period of 50 min at -35°C under nitrogen. After additional stirring for 30 min, excess hydride was decomposed by the addition of THF-water mixture at 0°C, and the reaction mixture was partitioned between ethyl acetate and water. The organic layer was washed with a saturated NaCl solution, dried over Na_2SO_4 and evaporated under reduced pressure to give a slightly yellow oil which was used without any purification for the next step. A part of the oil was purified by preparative TLC for measurement of ^{1}H-NMR spectrum.

^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 1.18(3H,t,J=7.2 Hz, $-OCH_2\underline{CH_3}$), 1.48(3H, d,J=5.3 Hz, $-CH\underline{CH_3}$), 3.85(6H,s,Ar-OCH_3), 3.52 - 4.16(2H,m,$-O\underline{CH_2}CH_3$), 4.30(2H, d,J=5 Hz, γ-CH_2-), 5.24(1H,q,J=5.3 Hz, $-\underline{CH}CH_3$), 6.24(1H,dd,J=15.5, 5 Hz,β-CH-), 6.54(1H,d,J=15.5 Hz, α-CH-), 6.58(2H,s,Ar-H).

d,l-Syringaresinol(V)

Ethoxyethyl sinapyl alcohol(III) obtained above was dissolved in a mixture of THF-water(9 : 1, 10 ml), and pyridinium *p*-toluenesulfonate(778 mg, 2.5 mmol) was added. After stirring for 70 min at room temperature under nitrogen, III was almost converted to sinapyl alcohol(IV) as judged by TLC.

The reaction mixture was then added to a stirred solution of horseradish peroxidase(8 mg, Crude; Sigma) in distilled water(500 ml), and 70 ml of 1% H_2O_2 was added slowly to the solution. After stirring for 4 h at room temperature, the solution was saturated with NaCl and extracted with four-150 ml portions of methylene chloride. The organic layer was washed with a saturated NaCl solution, dried over Na_2SO_4 and evaporated under reduced pressure to give a crude product from which 907 mg of crude crystals of V were separated. The residue was subjected to column chromatography(Wako-gel C-100, 120 g ; 3.5 × 21 cm) eluted with 2.5% methanol in methylene chloride. By this procedure, 2.784 g of V was obtained as slightly yellow crystals(overall yield from II was 66.5%).

Mp. 175 - 176°C. IR ν_{max}^{KBr} cm^{-1} : 3500, 2940, 2860, 1610, 1460, 1425, 1375, 1320, 1205, 1110. ^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 3.1(2H,m,β-CH-), 3.90(12H,s,Ar-OCH_3), 3.86 - 4.40(4H,m,γ-CH_2-), 4.73(2H,d,J=4 Hz, α-CH-), 5.59 (2H,s,Ar-OH), 6.57(4H,s,Ar-H). (acetate)(200 MHz, $CDCl_3$) : 2.34(6H,s,Ar-$OCOCH_3$), 3.10(2H,m,β-CH-), 3.83(12H,s,Ar-OCH_3), 3.95(2H,dd,J=9.2, 3.7 Hz,γ-CH_{axial}), 4.31(2H,dd,J=9.2, 6.9 Hz, γ-$CH_{equatorial}$), 4.77(2H,d,J=4.2 Hz, α-CH-), 6.59(4H,s,Ar-H). MS *m/z*(%) : 418(M^+, 85.6), 251(6.3), 235(12.6), 221 (10.8), 210(18.9), 208(11.7), 205(13.5), 193(32.4), 181(100), 175(9), 167 (92), 161(38.7), 154(30.6). (acetate) : 502(M^+, 2), 460(33.3), 418(100), 264(2), 251(3.6), 235(6.8), 221(4), 210(11.2), 209(9.2), 205(5.6), 193(17.7), 181(35.1), 167(36.1), 161(11.6), 154(9.6).

Syringaresinol monobenzyl ether(VI)

A mixture of benzyl chloride(80 μl, 0.7 mmol) and KI(116 mg, 0.7 mmol) in 8 ml of DMF(dried over molecular sieve 4A) was stirred for 30 min at room temperature under nitrogen. *d,l*-Syringaresinol(209 mg, 0.5 mmol) and NaH(24 mg, 1 mmol) were added to the solution, and the mixture was warmed

up to 50°C and stirred for additional 6 h. The reaction mixture was then poured onto water(30 ml), acidified to pH 4 with 1 N HCl and extracted four-60 ml portions of ethyl ether. The organic layer was washed with a saturated NaCl solution, dried over Na_2SO_4 and evaporated under reduced pressure to give a brown residue which was subjected to preparative TLC(ethyl acetate : *n*-hexane, 4 : 5, v/v) yielding 122.7 mg of the expected monobenzyl ether(VI, 48.2%), 51.7 mg of dibenzyl ether(17.2%) and 64 mg of starting material.

^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 3.1(2H,m,β,β'-CH-), 3.85(6H,s) and 3.90 (6H,s)(Ar-OCH_3), 3.89 - 4.17(4H,m,γ,γ'-CH_2-), 4.74(2H,br d,J=3 Hz, α,α'-CH-), 4.99(2H,s,Ar-CH_2-), 5.61(1H,s,Ar-OH), 6.57(4H,s,Ar-H) and 7.19 - 7.56(5H,m, Ar-H).

α-*Phenoxy acetate(VII)*

A mixture of monobenzyl ether(VI)(105 mg, 0.21 mmol), K_2CO_3(30.4 mg, 0.22 mmol), KI(36.5 mg, 0.22 mmol) and $ClCH_2COOCH_3$(19 μl, 0.22 mmol) in 6.5 ml of acetone was stirred overnight at reflux temperature. After cooling to room temperature, inorganic salts were filtered off and washed with acetone. The combined filtrate and washings were evaporated under reduced pressure to give a residue which was dissolved in ethyl acetate. The ethyl acetate solution was washed with a saturated NaCl solution, dried over Na_2SO_4. Removal of the solvent gave a crude syrap of VII which was purified by preparative TLC(ethyl acetate : *n*-hexane, 1 : 1, v/v) to yield 115.3 mg of VII (96.1%).

IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1} : 1770. ^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 3.1(2H,m,β, β'-CH-), 3.79(3H,s,-$COOCH_3$), 3.83(6H,s) and 3.86(6H,s)(Ar-OCH_3), 3.92 - 4.43 (4H,m,γ,γ'-CH_2-), 4.60(2H,s,-CH_2-), 4.73(2H,d,J=4 Hz, α,α'-CH-), 4.98(2H,s, Ar-CH_2-), 6.54(4H,s,Ar-H), 7.26 - 7.56(5H,m,Ar-H).

β-Hydroxy ester(VIII)

To a stirred solution of diisopropylamine(42 μl, 0.3 mmol) distilled from sodium metal in 9 ml of anhydrous THF(freshly distilled from benzophenone and potassium metal) were added 157 μl(0.225 mmol) of *n*-butyl lithium hexane solution(1.43 N, titrated in the usual way[11] before use) at -72 °C over a period of 6 min under nitrogen and the stirring was continued for additional 30 min at the same temperature. A solution of compound VII(109 mg, 0.188 mmol) in 3 ml of anhydrous THF was added dropwise to the mixture over a period of 40 min with stirring, and after additional 30 min stirring 90.8 mg(0.375 mmol) of benzyl vanillin[9] in anhydrous THF(3 ml) was added dropwise to the mixture over a period of 30 min. Stirring was continued for additional 1 h, then crushed dry-ice was added to the reaction mixture which was partitioned between ethyl acetate and a saturated NaCl solution. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to give a crude product which was purified by preparative TLC(ethyl acetate : *n*-hexane, 9 : 11, v/v) affording 98.7 mg of the β-hydroxy ester (VIIIe) and 11.4 mg of its isomer(VIIIt) in the total yield of 71.7%.

IR(mixture) ν_{max}^{KBr} cm^{-1} : 3520, 1750. ^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) *erythro*-isomer(VIIIe) : 2.99 - 3.18(2H,m,β',β"-CH-), 3.56(3H,s,γ-$COOCH_3$), 3.83 (12H,s) and 3.87(3H,s)(Ar-OCH_3), 3.92 - 4.44(4H,m,γ',γ"-CH_2-), 4.64 - 4.94 (4H,m,α,α',α",β-CH-), 4.96(2H,s)and 5.12(2H,s)(Ar-CH_2-), 6.54(4H,s) and 6.56 - 6.93(3H,m)(Ar-H), 7.21 - 7.57(10H,m,Ar-H). (acetate of VIIIe)(200 MHz, $CDCl_3$) : 2.02(3H,s,α-$OCOCH_3$), 3.06(2H,m,β',β"-CH-), 3.71(3H,s,γ-$COOCH_3$), 3.67(6H,s), 3.83(6H,s) and 3.88(3H,s)(Ar-OCH_3), 3.9 - 4.0(2H,m) and 4.23 - 4.33(2H,m)(γ',γ"-CH_2-), 4.69(1H,d,J=4.9 Hz) and 4.72(1H,d,J=4.7 Hz)(α',α"-CH-), 4.83(1H,d,J=6.3 Hz, β-CH-)*, 4.98(2H,s) and 5.14(2H,s)(Ar-CH_2-), 6.07 (1H,d,J=6.3 Hz, α-CH-), 6.46(2H,s) and 6.55(2H,s)(Ar-H on syringyl nuclei), 6.82(1H,d,J=8.3 Hz), 6.92(1H,dd,J=8.3, 1.8 Hz) and 7.12(1H,br d,J=1.4 Hz)

(Ar-H on guaiacyl nucleus), 7.25 - 7.55(10H,m,Ar-H). *Threo*-isomer(VIIIt) (90 MHz, $CDCl_3$) : 2.98 - 3.19(2H,m,β',β"-CH-), 3.53(3H,s,γ-$COOCH_3$), 3.86(15H, s,Ar-OCH_3), 3.94 - 4.43(4H,m,γ',γ"-CH_2-), 4.69 - 4.99(4H,m,α,α',α",β-CH-), 4.99(2H,s) and 5.11(2H,s)(Ar-CH_2-), 6.56(4H,s) and 6.71 - 6.91(3H,m)(Ar-H), 7.21 - 7.57(10H,m,Ar-H). (acetate of VIIIt)(200 MHz, $CDCl_3$) : 2.05(3H,s,γ-$OCOCH_3$), 3.09(2H,m,β',β"-CH-), 3.52(3H,s,γ-$COOCH_3$), 3.77(6H,s), 3.83(6H,s) and 3.87(3H,s)(Ar-OCH_3), 3.9 - 4.0(2H,m) and 4.24 - 4.33(2H,m)(γ',γ"-CH_2-), 4.67(1H,d,J=7.3 Hz, β-CH-)*, 4.73(2H,m,α',α"-CH-), 4.98(2H,s) and 5.12(2H,s) (Ar-CH_2-), 6.16(1H,d,J=7.3 Hz, α-CH-), 6.52(2H,s) and 6.55(2H,s)(Ar-H on syringyl nuclei), 6.80(1H,d,J=8.3 Hz), 6.87(1H,dd,J=8.3, 1.7 Hz) and 6.92(1H, d,J=1.7 Hz) (Ar-H on guaiacyl nucleus), 7.25 - 7.55(10H,m,Ar-H). *Weak splittings were observed : *erythro*; 1.2 Hz and *threo*; 0.6 Hz.

Diol(IX)

To a stirred solution of $LiAlH_4$(21 mg, 0.558 mmol) suspended in anhydrous THF(3 ml), 75.3 mg(0.091 mmol) of VIII in anhydrous THF(3 ml) was added dropwise over a period of 30 min at 50°C. After additional stirring for 30 min, excess hydride was decomposed by the addition of THF-water mixture at 0°C. The reaction mixture was partitioned between ethyl acetate and water. The organic layer was washed with a saturated NaCl solution, dried over Na_2SO_4 and evaporated under reduced pressure to give a crude product which was purified by preparative TLC(ethyl acetate : *n*-hexane, 3 : 2, v/v) yielding 61.8 mg(85.4%) of diol derivative IX.

^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 2.99 - 3.22(2H,m,β',β"-CH-), 3.84(6H,s) and 3.88(9H,s)(Ar-OCH_3), 3.89 - 4.44(8H,m,α,β-CH- and γ,γ',γ"-CH_2-), 4.76 (2H,d,J=3.6 Hz, α',α"-CH-), 4.99(2H,s) and 5.11(2H,s)(Ar-CH_2-), 6.56(2H,s), 6.61(2H,s) and 6.74 - 7.02(3H,m)(Ar-H), 7.26 - 7.56(10H,m,Ar-H).

Guaiacylglycerol-β-syringaresinol ether(X)

To a stirred solution of diol IX(57.1 mg, 0.072 mmol) in a mixture of methanol and dioxane(3 : 1, 4 ml), 30 mg of 10% palladium/carbon was suspended. The mixture was stirred under hydrogen at room temperature for 1 h. Palladium/carbon was filtered off and washed with methanol. The combined filtrate and washings were evaporated under reduced pressure to give a crude product which was purified by preparative TLC(methanol : methylene chloride, 3 : 97, v/v) yielding 39.5 mg of pure trilignol X(88.9%).

Anal. Calcd. for $C_{38}H_{32}O_{12}\cdot\frac{1}{2}H_2O$ C ; 61.62, H ; 6.30 Found C ; 61.26, H ; 6.46. UV λ_{max}^{EtOH} nm(log ε) : 230(4.33, shoulder), 278.5(3.65). IR ν_{max}^{KBr} cm^{-1} : 3500, 3370, 2922, 2852, 1596, 1524, 1458, 1426, 1369, 1221, 1120. ^{1}H-NMR(tetraacetate)(90 MHz, $CDCl_3$) δ(ppm) : 1.98(3H,s, γ-$OCOCH_3$), 2.14(3H, s, α-$OCOCH_3$), 2.28(3H,s) and 2.33(3H,s)(Ar-$OCOCH_3$), 2.97 - 3.18(2H,m,β',β"-CH-), 3.77(6H,s), 3.82(3H,s) and 3.85(6H,s)(Ar-OCH_3), 3.95 - 4.50(6H,m,γ,γ', γ"-CH_2-), 4.56 - 4.83(3H,m,α',α",β-CH-), 6.11(1H,d,J=5 Hz, α-CH-), 6.52(2H,s) and 6.59(2H,s)(Ar-H on syringyl nuclei), 6.92 - 7.03(3H,m,Ar-H on guaiacyl nucleus). ^{13}C-NMR(tetraacetate)(acetone-d_6) δ(ppm) : 20.27, 20.47, 20.61 and 20.94(-OCO$\underline{C}H_3$), 55.33 and 55.43(β',β"-C), 56.23, 56.35 and 56.42(-OCH_3), 63.28(γ-C), 72.63(γ',γ"-C), 75.03(α-C), 81.25(β-C), 86.45 and 86.54(α',α"-C), 103.18, 103.67, 112.01, 123.25, 128.79, 135.44, 137.14, 138.90, 140.51, 141.45, 152.00, 153.21 and 154.03(Ar-C), 168.62, 168.95, 169.92 and 170.68 (-O$\underline{C}OCH_3$). MS(tetraacetate) *m/z*(%) : 782(M^+, 0.7), 502(5.8), 460(45.2), 418(100), 251(5.8), 235(11.1), 222(59.8), 210(11.1), 193(22.7), 181(48.1), 162(29.2), 151(23.3), 147(25.7), 137(16.6), 131(33.7), 119(40.8), 103(31.5).

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CHAPTER II

DEGRADATION OF *d,l*-SYRINGARESINOL AND ARYLGLYCEROL-β-SYRINGARESINOL ETHERS BY *FUSARIUM SOLANI* M-13-1

INTRODUCTION

Iwahara *et al.* isolated many strains of microorganisms including bacteria, yeasts and molds, which could grow on medium containing coniferyl alcohol DHP as a sole source of carbon, from various natural materials by enrichment culture techniques[1,2]. Of these, the mold identified as *Fusarium* sp. degraded DHP extensively. The research showed that Fusaria utilized the relatively low molecular weight fraction of DHP, and uncondensed units were effectively degraded.

Furthermore, Iwahara *et al.* found an enzyme participating in the conversion of cinnamyl alcohol group to the corresponding aldehyde[3]. They also showed that this extracellular and constituent enzyme oxidized such an end group in DHP as in the case of model dimer, dehydrodiconiferyl alcohol. Recent findings on the enzymes in *Fusarium* sp. has been reported by Iwahara[4].

In this Chapter, degradation of *d,l*-syringaresinol and arylglycerol-β-syringaresinol ethers by the soil fungus *Fusarium solani* M-13-1, which can degrade DHP most actively[1], was described and degradation pathways were proposed based on the degradation products identified spectroscopically.

Degradation of other types of lignin substructure model compounds by the fungus has been intensively studied[5]. *F. solani* can degrade phenolic dilignols, but cannot degrade non-phenolic models. Alkyl-aryl(C_α-C_1) bond

cleavage in the dimers has been characterized as a predominant degradative reaction by the fungus. Cinnamyl alcohol end group is oxidized by the fungus to the corresponding aldehyde and acid, and then cleaved to give aromatic aldehyde. Aromatic aldehyde is further oxidized or reduced to the acid or alcohol.

RESULTS

1. Degradation of *d,l*-Syringaresinol and Derivatives

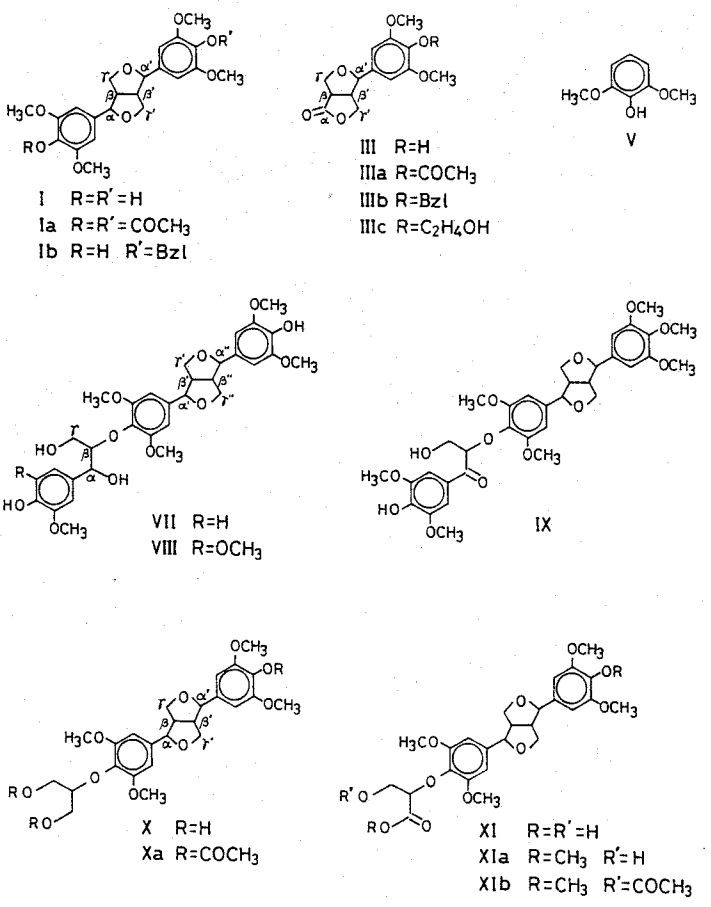

Fig. 1. Structures of compounds tested

Changes in UV-visible absorption spectra of d,l-syringaresinol during shake-culture

First, the effect of *Fusarium solani* M-13-1 on β-β' linked lignin substructure model compounds was examined using *d,l*-syringaresinol(I) in test tube cultures. The neutral and alkaline UV-visible spectra of the culture filtrates changed with incubation time as shown in Fig. 2. The increased absorbance at 306 nm and 368 nm(in alkaline solution) indicated that an α-carbonyl group conjugated with a *p*-hydroxy aromatic ring was being formed[6].

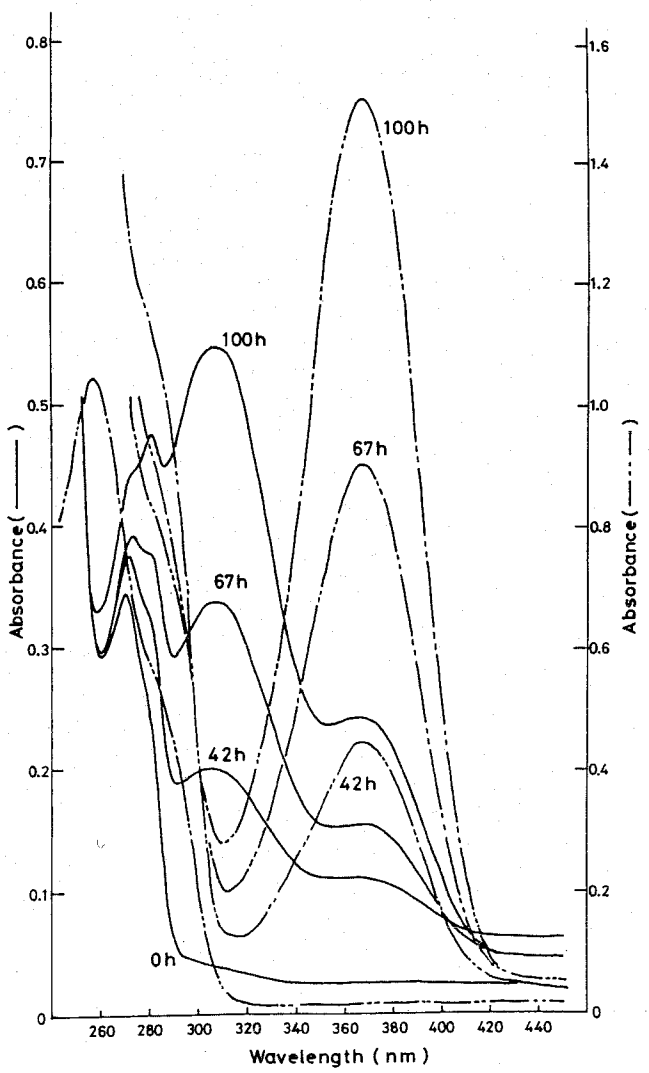

Fig. 2. Changes in UV-visible absorption of culture filtrates containing *d,l*-syringaresinol(I) during incubation with *Fusarium solani* M-13-1 : before(————) and after(——·——) addition of one drop of 1 N NaOH

The formation of an α-carbonyl group observed by the spectral analysis suggested that phenol-oxidizing enzymes(laccase and peroxidase) were involved in the degradation of I by the fungus. Similar spectral changes during oxidation of I with peroxidase/H_2O_2 have been reported by Pew *et al.*[7], and Caldwell and Steelink[8]. *d,l*-Syringaresinol dimethyl ether was not degraded by *F. solani*, indicating that free phenolic hydroxyl group is necessary for the degradation of this substructure. Based on these observations, degradation of syringaresinol monobenzyl ether(Ib) by *F. solani* and peroxidase - hydrogen peroxide system was further investigated.

Degradation of syringaresinol monobenzyl ether

UV-visible spectra of the culture filtrates containing Ib showed a similar pattern of changes as was seen in I. Fraction NE(see Experimental section) obtained from the culture filtrates after 52 h incubation was separated by preparative TLC to give four fractions(A,B,C and D). From fraction A, a yellow crystalline substance was obtained and identified as 2,6-dimethoxy-*p*-benzoquinone(IV) by comparison with the authentic sample by TLC, UV and MS. From fraction B, a white crystal, mp. 171 - 172 °C, was obtained and identified as 6-oxo-2-(4-benzyloxy-3,5-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane(IIIb) by comparison with the authentic sample by TLC, UV and MS. The yield of compounds IIIb and IV were 2.3% and 1.3%, respectively. Authentic specimens of IIIb and IV used were obtained as the oxidation products of Ib with peroxidase/H_2O_2(for details see Experimental section). Fraction C and D contained α-oxidized compound IIb* responsible for the characteristic spectral changes described above(Fig. 2). These were also characterized as a mixture of IIb-A and IIb-B by comparison with the compounds obtained by peroxidase oxidation of Ib. No compounds having free phenolic hydroxyl groups were detected on TLC except compounds Ib and IIb. Other

minor compounds seen on TLC were not examined.

Compounds IIIb and IV were also isolated from the culture filtrates after 145 h incubation as major products in the yields of 8.9% and 4.1%, respectively.

Degradation of d,l-syringaresinol

Low yields of the products from Ib might be partially due to its low solubility in the aqueous medium. In order to isolate substantial amounts of products from the cultures, further studies were made using *d,l*-syringaresinol(I). Compounds II*, III and IV were isolated from Fraction NE, and identified by ^{1}H-NMR, IR and MS. In addition, an unknown compound was isolated as the acetate. Compounds II-A and II-B were isolated as diacetate(II-Aa) and triacetate(II-Ba), respectively. The ^{1}H-NMR spectrum of II-Aa, obtained in a small amount, showed vague signals compared with that of II-Ba, and the structure was tentatively identified from its NMR and mass spectra referring to those of II-Ba. Acetylation of II-Aa with acetic anhydride - triethylamine in the presence of 4,4-dimethylaminopyridine(DMAP) gave II-Ba(TLC, NMR).

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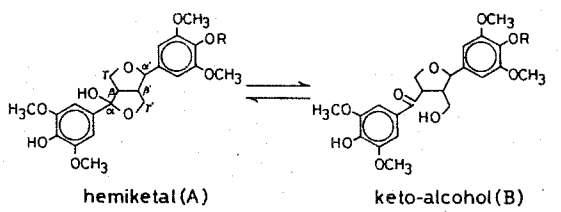
*The α-oxidized compounds(II and IIb) can occur in two chemical structures, namely, hemiketal(A) and keto-alcohol(B). The α-OH group in the hemiketal (A) was not acetylated(Cf. compound II-Aa). R=H(II), R=Bzl(IIb)



hemiketal(A) keto-alcohol(B)

Ring-chain tautomerism between the hemiketal(A) and keto-alcohol(B)

It seemed that most part of the mixture(II-A,B) gave the triacetate of keto-alcohol II-B, II-Ba, by the usual acetylation for 24 h. Therefore, acetylation is suitable to facilitate the isolation and structural elucidation of α-oxidized compounds derived from resinol structure.

Compound II-Aa. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 2.34(3H,s) and 2.35 (3H,s)(Ar-$OCOCH_3$), 3.16(1H,m,β'-CH-), 3.25(1H,m,β-CH-), 3.84(6H,s) and 3.86 (6H,s)(Ar-OCH_3), 3.8 - 4.0(2H,m,γ-CH_2-), 4.3 - 4.45(2H,m,γ'-CH_2-), 4.55(1H,d, J=6 Hz, α'-CH-), 6.61(2H,s) and 6.85(2H,s)(Ar-H). MS *m/z*(%) : 518(M^+, 10.4), 476(42.7), 458(5.7), 434(13), 400(6.2), 267(7.1), 252(4.7), 235(22.9), 226 (31.8), 208(39.6), 196(9.4), 181(100), 167(15.1), 153(9.9), 43(46.9).

Compound II-Ba. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 1.65(3H,s,γ'-$OCOCH_3$), 2.34(3H,s) and 2.35(3H,s)(Ar-$OCOCH_3$), 2.93(1H,m,β'-CH-), 3.85(6H,s) and 3.88 (6H,s)(Ar-OCH_3), 4.14(2H,d,J=7.4 Hz, γ'-CH_2-), 4.1 - 4.5(3H,m,β-CH- and γ-CH_2-), 4.79(1H,d,J=6.8 Hz, α'-CH-), 6.66(2H,s) and 7.21(2H,s)(Ar-H). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1} : 1678(α-C=O), 1750 and 1770(acetyl-C=O). MS *m/z*(%) : 560(M^+, 3.6), 518 (16.3), 476(4.8), 458(9.1), 441(2.9), 277(5.3), 268(10.1), 252(6.5), 235 (25.9), 209(29.5), 208(100), 182(11.5), 181(65.3), 167(8.6), 153(5.7), 43 (42.1).

Compound III. ^{1}H-NMR(acetate IIIa)(200 MHz,$CDCl_3$) δ(ppm) : 2.35(3H,s, Ar-$OCOCH_3$), 3.11(1H,m,β'-CH-), 3.45(1H,br dt,J=9.4, 3.8 Hz,β-CH-), 3.83(6H, s,Ar-OCH_3), 4.24(1H,dd,J=9.4, 3.8 Hz) and 4.40(1H,approx. t,J=9.4 Hz)(γ -CH_2-), 4.37(1H,dd,J=10, 2.2 Hz) and 4.53(1H,dd,J=10, 6.6 Hz)(γ'-CH_2-), 4.65(1H,d,J=7 Hz,α'-CH-), 6.59(2H,s,Ar-H). IR ν_{max}^{KBr} cm^{-1} : 1770(α-C=O). MS *m/z*(%) : 280 (M^+, 100), 265(4.9), 249(11.4), 237(5.1), 221(3.4), 210(5.7), 196(8.6), 195 (8.9), 183(20), 182(71.4), 181(48.9), 168(24.3), 167(93.7), 165(24.3), 155 (16.3), 153(12.6), 123(16.6).

Degradability of 2,6-dimethoxyphenol

To confirm the direct formation of *p*-quinone IV by oxidative cleavage

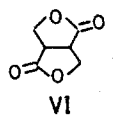
of I, the effect of *F. solani* M-13-1 on 2,6-dimethoxyphenol(V), which would be released from II-A via retro aldol type cleavage, was examined. After 30 h of incubation the visible spectrum of the culture filtrate showed a small absorption maximum at around 470 nm. A violet precipitate was observed in the culture medium after 40 h incubation, which was probably coerulignone, an insoluble violet compound[9, 10] ($\lambda_{max}^{CHCl_3}$: 470 nm). After 52 h incubation both Fractions NE and AE were examined, but *p*-quinone IV could not be obtained.

Degradation of γ-lactone derivatives

The γ-lactone derivatives, III and IIIb, were obtained as degradation products of *d,l*-syringaresinol(I) and its monobenzyl ether(Ib), respectively. Therefore, γ-lactone derivative IIIc having the same structural unit and increased solubility was prepared and used for examining the fungal degradability of the 2-aryl-6-oxo-3,7-dioxabicyclo[3,3,0]octane structure. UV spectra of the culture filtrates of IIIc showed no changes, and no products were detected on TLC, during shake-culture with *F. solani*.

However, γ-lactone III having a free phenolic hydroxyl group was found to be degraded by the fungus ; the UV-visible spectra of the culture filtrates showed a similar pattern of changes as was seen with I.

The UV spectra of the reaction mixture during oxidation of III with peroxidase/H_2O_2 also changed in a similar way as shown in Fig. 3. Products after 3.5 h were extracted and analyzed by gas chromatography - mass spectrometry(GC - MS). 2,6-Dimethoxy-*p*-benzoquinone(IV) and 2,6-dioxo-3,7-dioxabicyclo[3,3,0]octane(VI) were identified as degradation products of III by comparison with the authentic samples.



VI

Fraction NE obtained from the culture incubated with I was again examined for the presence of dilactone VI, but the compound could not be detected by TLC and GC.

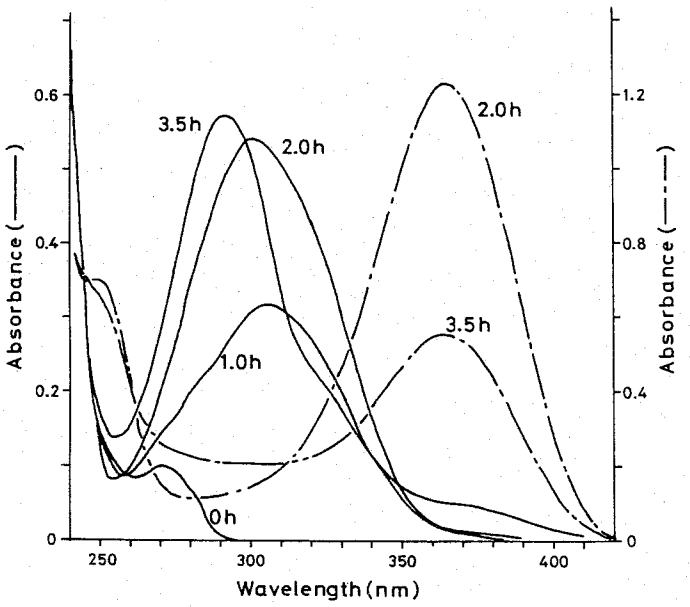


Fig. 3. Changes in UV-visible apsorption of γ-lactone derivative III during oxidation with peroxidase - hydrogen peroxide

2. Degradation of Arylglycerol-β-syringaresinol Ethers

Degradation of guaiacylglycerol-β-syringaresinol ether

When trilignol VII was incubated with *F. solani*, UV spectra of the culture filtrates showed the occurrence of a conjugated carbonyl group, which indicated the oxidation of a benzylic position(α or α") in the molecule as observed in the degradation of *d,l*-syringaresinol(I). This was confirmed by the isolation of the corresponding compounds(II and XII).

Fraction NE obtained after 72 h incubation was separated by repeated TLC to give five compounds I, II, IV, X and XII. Compounds I and IV were identified by comparison with the authentic samples by TLC and MS. Compounds II-A,B, X and XII were isolated as the corresponding acetates, and II-Aa, II-Ba and Xa were identified by comparison with the authentic samples

by TLC and MS. The structure of compound XIIa was determined from its ^{1}H-NMR and IR spectra which were assigned as follows referring to those of compound II-Ba.

Compound XIIa. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 1.64(3H,s,γ'-$OCOCH_3$), 1.99(3H,s,γ-$OCOCH_3$), 2.14 and 2.15(3H,two s,α-$OCOCH_3$), 2.29(3H,splitted s) and 2.35(3H,s)(Ar-$OCOCH_3$), 2.88(1H,m,β'-CH-), 3.76, 3.77, 3.81, 3.82, 3.88 and 3.90(15H,six s,Ar-OCH_3), 4.10(2H,d,J=7.6 Hz, γ'-CH_2-), 4.2 - 4.55(5H,m, β"-CH- and γ,γ"-CH_2-), 4.62(1H,m,β-CH-), 4.75(1H,d,J=7.4 Hz, α'-CH-), 6.06 - 6.12(1H,m,α-CH-), 6.58(2H,s), 6.86 - 7.12(3H,m) and 7.22(2H,s)(Ar-H). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1} : 1678(α"-C=O), 1750 and 1765(acetyl-C=O).

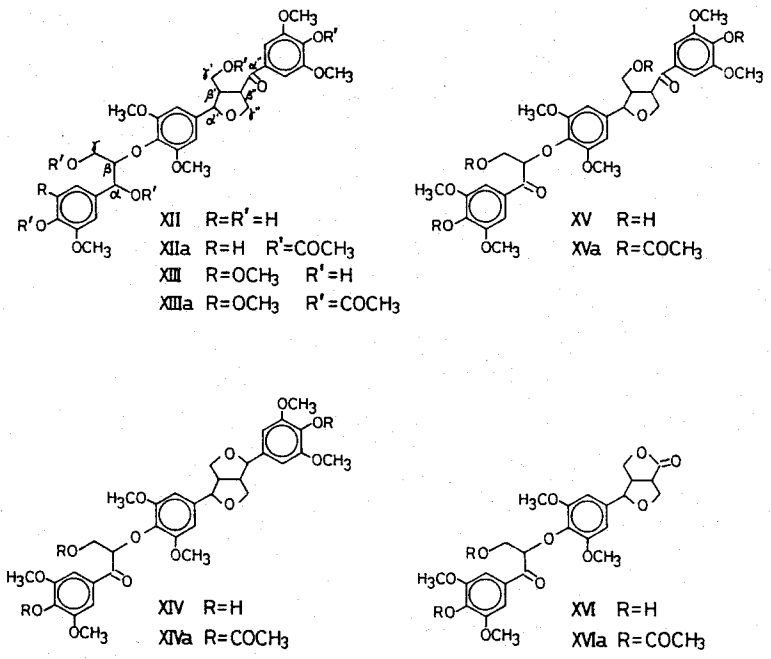


Fig. 4. Structures of identified compounds

In addition, a compound assumed to be the stereoisomer of XII between the β' and β" positions, was isolated as the acetate XII'a ; probably XII has *cis* and XII' has *trans* configuration.

Compound XII'a. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 1.96(3H,s,γ'-$OCOCH_3$), 2.00(3H,s,γ-$OCOCH_3$), 2.18(3H,s,α-$OCOCH_3$), 2.30(3H,s) and 2.38(3H,s)(Ar-$OCOCH_3$), 3.07(1H,m,β'-CH-), 3.82, 3.89 and 3.91(15H,three s,Ar-OCH_3), 4.06

(1H,m,β"-CH-), 4.14 - 4.56(6H,m,γ,γ',γ"-CH_2-), 4.56 - 4.70(1H,m,β-CH-), 4.61 (1H,d,J=8.6 Hz, α'-CH-), 6.10(1H,m,α-CH-), 6.63(2H,s), 6.9 - 7.1(3H,m) and 7.23(2H,s)(Ar-H).

The presence of compounds I, II, IV, X and XII clearly indicated that oxidation at the α"-position and cleavage of alkyl-aryl bonds(C_α-C_1 and $C_{\alpha"}$-$C_{1"}$) and splitting of the β-aryl ether bond(C_β-*O*-$C_{4'}$) occurred at some stage.

Fraction AE was methylated with diazomethane and acetylated sucessively, and purified by preparative TLC. Compound XIb, methylated and acetylated derivative of glyceric acid-2-syringaresinol ether(XI), was isolated and identified by comparison with the authentic sample by TLC, ^{1}H-NMR and MS. This also indicated the alkyl-aryl cleavage of VII.

Since compounds X and XI were obtained as degradation products, further degradation of these intermediates was examined with special interest in β-ether cleavage. However, both compounds did not give the products expected(I or II) by β-aryl ether cleavage in detectable amounts. Compounds analogous to II with a glycerol or glyceric acid moiety were obtained in both cases. The results indicated that X and XI were degraded only at the syringaresinol portion bearing free phenolic hydroxyl group.

Degradation of syringylglycerol-β-syringaresinol ether

Fraction NE obtained after 60 h incubation of trilignol VIII was acetylated and separated by repeated preparative TLC to give six compounds Ia, IV, XIIIa, XIVa, XVa and XVIa. The substrate VIII was recovered in about 26% yield. Compound XIIIa was readily identified from its ^{1}H-NMR spectrum by comparison with that of previously identified guaiacyl analog XIIa.

Compound XIIIa. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 1.64(3H,s,γ'-$OCOCH_3$), 2.01(3H,s,γ-$OCOCH_3$), 2.16(3H,splitted s,α-$OCOCH_3$), 2.32(3H,s) and 2.36(3H,

s)(Ar-$OCOCH_3$), 2.89(1H,m,β'-CH-), 3.765, 3.77, 3.785, 3.80 and 3.875(18H, five s,Ar-OCH_3), 4.10(2H,d,J=7.2 Hz, γ'-CH_2-), 4.2 - 4.55(5H,m,β"-CH- and γ, γ"-CH_2-), 4.63(1H,m,β-CH-), 4.75(1H,d,J=7.5 Hz, α'-CH-), 6.1(1H,m,α-CH-), 6.62(2H,s), 6.67(2H,s) and 7.23(2H,s)(Ar-H). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1} : 1680(α"-C=O), 1750 and 1765(acetyl-C=O).

Compound XIVa was identified by ^{1}H-NMR, IR and MS. The NMR and IR spectra were assigned as follows referring to the data of IX.

Compound XIVa. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 1.99(3H,s,γ-$OCOCH_3$), 2.35(3H,s) and 2.36(3H,s)(Ar-$OCOCH_3$), 3.08(2H,m,β',β"-CH-), 3.74, 3.83 and 3.86(18H,three s,Ar-OCH_3), 3.9 - 4.0(2H,m) and 4.30(2H,br dd,J=8.5, 7.5 Hz) (γ',γ"-CH_2-), 4.50(1H,dd,J=11.8, 6.2 Hz) and 4.61(1H,dd,J=11.8, 4.8 Hz)(γ-CH_2-), 5.46(1H,dd,J=6.2, 4.8 Hz,β-CH-), 6.54(2H,s), 6.60(2H,s), 7.50(1H,s) and 7.51(1H,s)(Ar-H). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1} : 1685(α-C=O), 1750 and 1765(acetyl-C=O). MS *m/z*(%) : 708(M^+ - CH_3COOH, 1.2), 666(1.5), 624(0.8), 460(37.5), 418(100), 309(8.9), 266(6.3), 251(6.3), 235(12.6), 210(19), 208(27.8), 193 (29.9), 181(98.7), 167(70.4), 161(23.8), 155(19), 60(42.5), 43(74.4). This compound was also found to be formed in the control culture without mycelia after prolonged incubation(145 h) with about 1% yield.

The structure of compound XVa was characterized by ^{1}H-NMR, IR and MS. The NMR and IR spectra were assigned as follows referring to the data of compounds II-Ba and IX.

Compound XVa. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 1.64(3H,s,γ'-$OCOCH_3$), 2.00(3H,splitted s,γ-$OCOCH_3$), 2.36(6H,s,Ar-$OCOCH_3$), 2.88(1H,m,β'-CH-), 3.76 and 3.87(18H,two s,Ar-OCH_3), 4.11(2H,d,J=7.2 Hz, γ'-CH_2-), 4.2 - 4.7(5H,m,β"-CH- and γ,γ"-CH_2-), 4.75(1H,d,J=7.5 Hz, α'-CH-), 5.48(1H,m,β-CH-), 6.60(2H, s), 7.20(2H,s), 7.51(1H,s) and 7.52(1H,s)(Ar-H). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1} : 1675 (α"-C=O), 1688(α-C=O), 1750 and 1768(acetyl-C=O). MS *m/z*(%) : 766(M^+, 0.7), 742(0.8), 518(26.4), 476(11.9), 414(5.5), 252(28.4), 208(80.7), 196(9),

181(100), 167(14.9), 153(11.1), 60(13.4), 55(11.4), 45(25.5), 43(66).

The structure of compound XVIa was deduced from its ^{1}H-NMR and mass spectra referring to the data of compounds III and XIVa.

Compound XVIa. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 2.00(3H,s,γ-$OCOCH_3$), 2.36(3H,s,Ar-$OCOCH_3$), 3.10(1H,m,β'-CH-), 3.44(1H,br dt,J=9, 4 Hz,β"-CH-), 3.76(6H,s) and 3.86(6H,s)(Ar-OCH_3), 4.18 - 4.63(7H,m,α'-CH- and γ,γ',γ"-CH_2-), 5.48(1H,dd,J=7.5, 4 Hz,β-CH-), 6.52(2H,s), 7.47(1H,s) and 7.48(1H,s)(Ar-H). MS *m/z*(%) : 588(M^+, trace), 528(4.5), 486(4.5), 309(22.3), 291(5.3), 280 (23.6), 267(7.7), 239(5.5), 208(22.3), 197(6), 185(8.9), 182(21.5), 181 (100), 167(20.9), 165(15), 153(8.3), 60(12.6), 55(16.6), 43(60.6).

Since compounds with an α-carbonyl group were obtained as intermediate degradation products, compound IX was used as a substrate for further investigation. As a main product compound XIa, dimethylated derivative of glyceric acid-2-syringaresinol ether(XI), was isolated from the methylated Fraction AE. The structure was identified by comparison with the synthetic standard by TLC, ^{1}H-NMR and MS. However, no compounds indicating the cleavage of the β-ether bond were found in Fraction NE in this case. Degradation of IX was slower than VII and VIII under the conditions used. These results proved oxidation at the α-position of the arylglycerol moiety and subsequent splitting of the alkyl-aryl(C_α-C_1) bond leading to the glyceric acid ether XI.

DISCUSSION

1. Degradation of *d,l*-Syringaresinol

Fusarium solani M-13-1 actively degraded *d,l*-syringaresinol(I). Figure 5 shows a proposed degradation pathway for the phenolic model I or Ib

by the fungus. The model phenol is first converted to the hemiketal II-A, 2-hydroxy-2,6-bis(4-hydroxy-3,5-dimethoxyphenyl)-3,7-dioxabicyclo[3,3,0]-octane, via disproponation of the radicals from I by the action of phenol-oxidizing enzymes to the quinonemethide, and subsequent hydration of the double bond. Hemiketal II-A can be transformed to the keto-alcohol II-B, 3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-4-(4-hydroxy-3,5-dimethoxybenzoyl)tetrahydrofuran, due to the ring - chain tautomerization. Further oxidation of II yields the unstable cyclohexadienone derivatives depicted in brackets ; origin of the C_1-OH is unknown. The speculative intermediates immediately give the γ-lactone derivative, 6-oxo-2-(4-hydroxy-3,5-dimethoxyphenyl)-3,7-dioxabicyclo[3,3,0]octane(Ⅲ), with release of 2,6-dimethoxyhydroquinone. The hydroquinone, which was not isolated, is oxidized to 2,6-dimethoxy-*p*-benzoquinone(IV) by phenol-oxidizing enzymes. Peroxidase oxidation of methoxyhydroquinone to the corresponding *p*-quinone has been demonstrated by Krisnangkura and Gold[11]. It is probable that

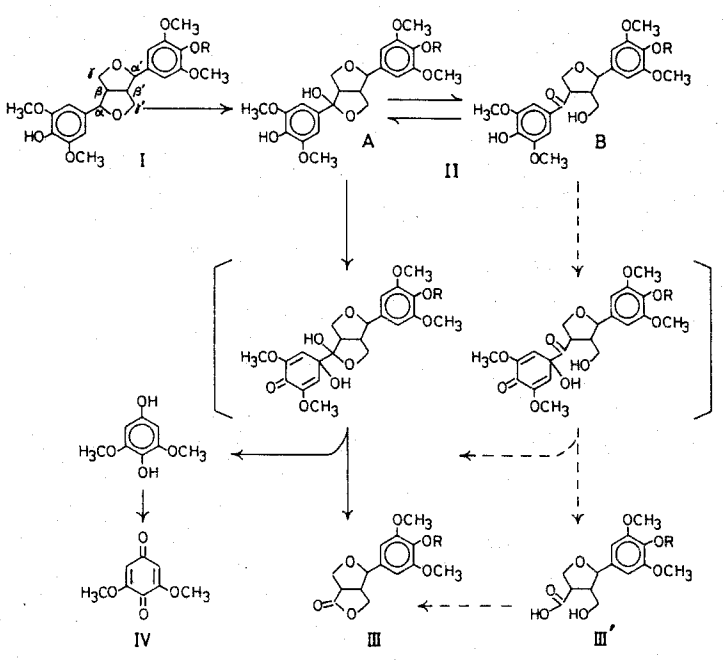


Fig. 5. Proposed degradation pathway for *d,l*-syringaresinol(I, R=H) and its monobenzyl ether(Ib, R=Bzl) by *Fusarium solani* M-13-1

the hydroquinone formed by the cleavage reaction is metabolized via ring cleavage.

Toms and Wood reported the degradation of α-conidendrin by *Pseudomonas multivorans* and proposed a retro aldol type cleavage for the splitting of the aromatic ring from this lignan[12]. If this type of non-oxidative alkyl-aryl cleavage had occurred in the degradation of I or Ib, 2,6-dimethoxy-phenol(V) would have been formed. However, the phenol was not found in the culture of *F. solani* incubated with I, and addition of the phenol to fungal culture did not give *p*-quinone IV. Compound V is quantitatively dimerized to coerulignone, 3,3',5,5'-tetramethoxydiphenoquinone by the phenol-oxidizing enzymes[9,10]. Accordingly, syringaresinol I was oxidatively degraded as described above to give compounds III and IV.

The main degradation products of compound I by *F. solani* M-13-1 were essentially the same as its oxidation products with peroxidase/H_2O_2 system. In addition, it was found that the fungus could not degrade syringaresinol dimethyl ether. These results, together with changes in the UV-visible spectra of the culture filtrates, indicate that the degradation of I by *F. solani* proceeds mainly via alkyl-aryl cleavage as depicted in Fig. 5. No products suggesting the direct C_α-C_β side chain cleavage were found in all cases. It is, however, noted that γ-lactone III could not be found during oxidation of I with peroxidase/H_2O_2.

The alkyl-aryl cleavage by microorganisms was studied earlier by Kirk *et al.* using a lignin substructure model compound, syringylglycol-β-guaiacyl ether. Cleavage was found to occur with formation of guaiacoxyacetoaldehyde and 2,6-dimethoxy-*p*-benzoquinone, by whole cultures of *Stereum frustlatum* and by culture filtrate(laccase) of *Polyporus versicolor*[13].

It was earlier found by Freudenberg *et al.* that prolonged dehydrogenation of sinapyl alcohol leads to decomposition of *d,l*-syringaresinol

initially formed, yielding *p*-quinone IV[14]. Present studies indicate that *d,l*-syringaresinol(I) could be finally degraded to *p*-quinone IV and *d,l*-dilactone VI by phenol-oxidizing enzyme activity of the fungus.

Recently, Iwahara and his co-workers[4] found that a laccase type enzyme was responsible for oxidative degradation of I by *Fusarium* sp.[15] and that another enzyme different from peroxidase and laccase was involved in the oxidation of *d,l*-pinoresinol by *Fusarium* sp.[16] The latter enzyme is also operative for oxidation of α-position of I[17].

2. Degradation of Arylglycerol-β-syringaresinol Ethers

Degradation studies on dilignols were extended to trilignols which contained two types of linkages(β-β' and β-*O*-4') ; guaiacylglycerol-β-syringaresinol ether(VII) and syringylglycerol-β-syringaresinol ether(VIII). After 3 days incubation, approximately 70% of both substrates was converted to several compounds identified with many unknown compounds detected on TLC plates. From the ^{1}H-NMR spectra, the *erythro/threo* ratios of the substrates recovered were found to be almost equal to those of the added ones, which suggested that both isomers were degraded without stereospecificity.

Based on the products identified, the degradation sequence for arylglycerol moieties of VII and VIII is proposed as shown in Fig. 6. The cleavage of alkyl-aryl(C_{α}-C_{1}) bond was indicated by the isolation of glycerol-2-syringaresinol ether(X, type[B] in Fig. 6) and glyceric acid-2-syringaresinol ether(XI, type[C]). This result is in good accord with the previous work on the β-ether dilignols[18]. Pew and Conners[19] demonstrated the alkyl-aryl cleavage of guaiacylglycerol-β-guaiacyl ether by peroxidase and hydrogen peroxide, giving glyceraldehyde-2-guaiacyl ether(type [A]). The glyceraldehyde ether[A] was not isolated in the present investigation, but the aldehyde intermediate[A] formed by the cleavage reaction is thought

to be reduced or oxidized to the corresponding alcohol[B] or acid[C], respectively by the fungus. Formation of analogous alcohol and acid from the β-ether dimer by *P. chrysosporium* has been reported[20]. The glyceric acid[C] was found to be formed by the fungus from the α-keto derivative[D], alternatively : the α-keto derivative has not been obtained from a dimeric β-ether model[18]. In contrast to the result with VIII, substrate VII did not give detectable amounts of an α-keto compound[D]. Thus the major degradation pathway of VII seems to be VII → [A] →([B] or [C]) as in the dilignol[18], although the degradation of VII via [D] cannot be excluded. The assumed counterpart product after cleavage reaction, methoxyhydroquinone or methoxy-*p*-quinone(R=H in Fig. 6), could not be isolated in the case of VII. This is probably due to the rapid metabolism or polymerization of the products.

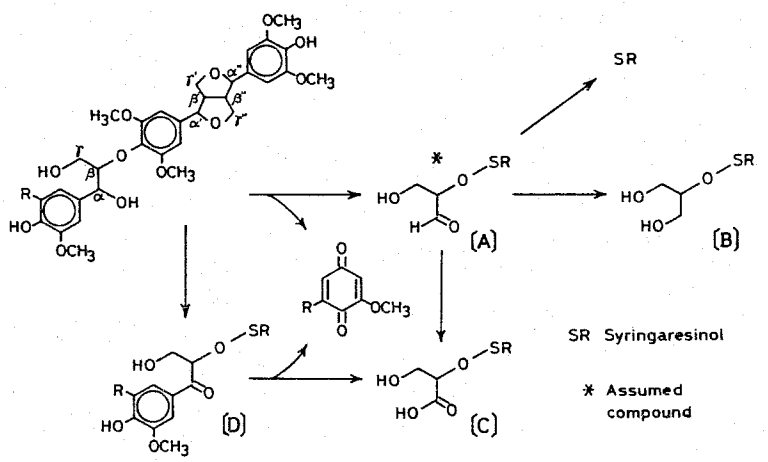


Fig. 6. Proposed degradation pathway for the arylglycerol moiety of compounds VII(R=H) and VIII(R=OCH_3) by *Fusarium solani* M-13-1

The isolation of *d,l*-syringaresinol(I) and its oxidation product(II) suggested the involvement of β-aryl ether cleavage. Splitting of the β-ether bond is important for breakdown of lignin macromolecule, because this substructure is most abundant in lignin. Enoki *et al.*[21] recently found the cleavage of the β-ether bond in 4-ethoxy-3-methoxyphenylglycerol-β-

guaiacyl ether by *P. chrysosporium* with formation of 4-ethoxy-3-methoxyphenylglycerol and guaiacol : the cleavage mechanism has not been established. However, the probable intermediates, arylglycerol or related derivatives, due to the β-ether cleavage of VII and VIII were not detected in the present investigation. In addition, no β-ether cleavage products were isolated from the degradation intermediates([B] and [C]) under the culture conditions used, although Katayama *et al.* found the formation of vanillic acid from glycerol-2-vanillic acid ether(type[B]) by *F. solani* (unpublished result). Crawford *et al.*[22] found that β-ether cleavage of guaiacylglycerol-β-guaiacyl ether by *Pseudomonas acidovorans* required the oxidation of the α-position as a prerequisite. The pathway was not found to be involved in *F. solani*.

It seems unlikely that products I and II are released on initial β-ether cleavage by the fungus. It seems that certain unknown intermediates such as glyceraldehyde ether[A] or others derived from [A] by the initial alkyl-aryl cleavage are involved in the β-ether cleavage. The presumable intermediate[A] may be important in the β-ether cleavage by *F. solani*.

On the other hand, C_α-C_β side chain cleavage in the etherated β-ether dilignol by *P. chrysosporium* has been reported[23]. The C_α-C_β cleavage seems to be the major degradative reaction of etherated moieties in white-rot fungi[24], but alkyl-aryl cleavage seems to be a predominant reaction of phenolic moieties[5].

Oxidation of the benzylic position, and subsequent splitting of the alkyl-aryl C-C bond in the syringaresinol moiety with a phenolic hydroxyl group were also indicated by the isolation of compounds IV, XII, XIII, XV and XVI. This was also consistent with the results of dimer model I. It was again confirmed that free phenolic hydroxyl group was necessary for the degradation of resinol substructure by *F. solani* M-13-1.

EXPERIMENTAL

Organism

Fusarium solani M-13-1 isolated from soil by an enrichment culture method using DHP as sole source of carbon[1] was used. The fungus was maintained on nutrient agar slants at 10°C.

Media and Culture Conditions

The basal inorganic medium contained the following salts(per liter) : NH_4NO_3, 2 g ; K_2HPO_4, 1 g ; KCl, 0.5 g ; $MgSO_4 \cdot 7H_2O$, 0.5 g ; $CaCl_2$, 20 mg ; $FeSO_4 \cdot 7H_2O$, 10 mg ; $MnCl_2 \cdot 4H_2O$, 5 mg ; $CuSO_4 \cdot 5H_2O$, 1 mg. The medium was adjusted to pH 6 with 1 N HCl before autoclaving. The nutrient medium used for fungal growth contained 20 g glucose, 5 g peptone and 1 g yeast extract per liter of the above medium.

The fungus was grown in a 500 ml Sakaguchi flask containing 100 ml of the nutrient medium with reciprocal shaking for 2 days at 29 - 30°C. Mycelia collected by filtration were washed with sterile inorganic medium, resuspended in the same salts medium and used in the degradation experiments.

Preliminary examination for fungal degradation was carried out in test tube cultures containing 5 ml of the inorganic medium, about 10 mg of washed mycelia and 2 μmol of each substrate. The supernatant was periodically taken and UV-visible spectra were measured after appropriate dilution.

Degradation experiment for isolation of the products was performed in 500 ml Sakaguchi flasks containing 50(for trimer) or 100(for dimer) ml of the basal salts medium, about 0.5(for dimer) or 1.2(for trimer) g of mycelia and 25(trimer) or 50(dimer) mg of substrates. Each substrate was added as DMF(0.5 - 1 ml) solution to the cultures.

Extraction and Isolation of Degradation Products

The degradation process was monitored by UV-visible spectroscopy and TLC of the culture filtrates. When compounds different from substrate were detected on a silica gel TLC plate under a UV light(254 nm) and with spray reagents($FeCl_3$ - $K_3Fe(CN)_6$, 2,4-dinitrophenylhydrazine - HCl, 2,6-dichloroquinone-4-chloroimine + 1 N NaOH), mycelia were removed by filtration and washed with distilled water. The combined filtrate and washings were extracted three times with ethyl acetate(Fraction NE). The aqueous layer was then acidified with conc. HCl at pH 2 and similarly extracted(Fraction AE). Both organic layers were washed with a saturated NaCl solution, dried over Na_2SO_4 and evaporated under reduced pressure at 40°C.

Products were separated and purified by preparative TLC(Kieselgel 60 F_{254}, Merck) using appropriate mixture of the following solvent systems : methanol - methylene chloride, ethyl acetate - *n*-hexane, methylene chloride - *n*-hexane, ethyl acetate - benzene.

Substrates and Intermediates

Compounds I and Ib. *d,l*-Syringaresinol(I) and its monobenzyl ether (Ib) were synthesized as described in Chapter I.

Compound IIIc. This compound was prepared by peroxidase oxidation of 4-*O*-(2-hydroxyethyl)-syringaresinol, which was synthesized by reduction of 4-*O*-benzyl-4'-*O*-carbomethoxymethyl-syringaresinol(a synthetic intermediate VII for trilignol, see Chapter I), with $LiAlH_4$ and H_2—Pd/C. Peroxidase oxidation was carried out as described later. The compound was purified by preparative TLC. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 3.12(1H,m,β'-CH-), 3.47(1H,br dt,J=4, 9 Hz,β-CH-), 3.82(2H,br t,J=4.5 Hz,-CH_2-), 3.89(6H,s,Ar-OCH_3), 4.13(2H,br t,J=4.5 Hz,-CH_2-), 4.23(1H,dd,J=9, 10 Hz) and 4.42(1H,br t,J=9 Hz) (γ-CH_2-), 4.37(1H,dd,J=10, 2 Hz) and 4.54(1H,dd,J=10, 9 Hz)(γ'-CH_2-), 4.63

(1H,d,J=7 Hz, α'-CH-), 6.58(2H,s,Ar-H). IR ν_{max}^{KBr} cm^{-1} : 1760(α-C=O). MS *m/z*(%) : 324(M^+, 58.2), 280(79.1), 265(6.4), 251(10.2), 249(12.8), 237(7.7), 196(14.3), 195(8.9), 183(25.5), 182(79.6), 181(47.2), 168(28.1), 167(100), 165(28.1).

Compound V. 2,6-Dimethoxyphenol(V) was commercially available(Tokyo Chemical Industry Co., Ltd.) and used after recrystallization; mp. 54.5°C.

Compounds VII and VIII. Arylglycerol-β-syringaresinol ethers VII and VIII (*erythro/threo* mixture) were synthesized as described in Chapter I. The structures were confirmed by ^{1}H-NMR.

Compound IX. This compound was synthesized according to the method described for the synthesis of 2-(4-formyl-2,6-dimethoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone[25] from 4-acetoxy-3,5-dimethoxy-α-bromoacetophenone and syringaresinol monomethyl ether. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 3.11(2H,m,β',β"-CH-), 3.76, 3.84, 3.88 and 3.92(21H, four s,Ar-OCH_3), 3.84 - 4.08(4H,m,γ,γ',γ"-CH_2-), 4.31(2H,br dd,J=8.8, 6.4 Hz, γ',γ"-CH_2-), 4.75(2H,m,α',α"-CH-), 5.07(1H,dd,J=3, 7.4 Hz,β-CH-), 6.14(1H,br s,Ar-OH), 6.57(2H,s), 6.58(2H,s) and 7.41(2H,s)(Ar-H). IR ν_{max}^{KBr} cm^{-1} : 1685 (α-C=O). UV λ_{max}^{EtOH} nm : 286(shoulder) and 310. (+NaOH) : 253.5(shoulder) and 372.8.

Compound X. To a stirred solution of syringaresinol monobenzyl ether (1.09 mmol) and NaH(1.1 mmol) in DMF(7 ml) was added dropwise diethyl bromomalonate(1.1 mmol) in DMF(3 ml) at -5°C. After stirring for an additional 40 min, the reaction mixture was poured onto ice-water, acidified with 1 N HCl and extracted three times with ethyl ether. The ethereal layer was washed with a saturated NaCl solution, dried over Na_2SO_4 and evaporated, resulting in a residue which was purified by TLC(ethyl acetate — *n*-hexane, 3 : 4, v/v, × 5) to give diethyl malonate-2-(syringaresinol monobenzyl ether) ether with about 70% yield. The malonate ether was converted to glycerol

ether by $LiAlH_4$ reduction in THF at 50°C and its benzyl protecting group was removed by catalytic hydrogenation on 10% Pd/C in methanol. After purification by preparative TLC(methanol - methylene chloride, 1 : 24, v/v), glycerol-2-syringaresinol ether(X) was obtained with about an 80% overall yield from the malonate ether. ^{1}H-NMR(200 MHz, $CDCl_3$) δ(ppm) : 3.10(2H,m, β,β'-CH-), 3.74(2H,dd,J=12, 3.8 Hz) and 3.83(2H,dd,J=12, 5.8 Hz)(-$C\underline{H}_2$OH), 3.90 (6H,s) and 3.91(6H,s)(Ar-OCH_3), 3.88 - 3.96(2H,m) and 4.30(2H,br dd,J=7, 9 Hz) (γ,γ'-CH_2-), 4.75(1H,d,J=4 Hz) and 4.755(1H,d,J=4.2 Hz)(α,α'-CH-), 5.54(1H, br s,Ar-OH), 6.58(2H,s) and 6.61(2H,s)(Ar-H). (triacetate Xa) : 2.08(6H,s, -$OCOCH_3$), 2.34(3H,s,Ar-$OCOCH_3$), 3.10(2H,m,β,β'-CH-), 3.83(12H,s,Ar-OCH_3), 3.89 - 4.01(2H,m,γ,γ'-CH_2-), 4.25 - 4.45(7H,m,γ,γ'-CH_2- and Ar-O$C\underline{H}$($C\underline{H}_2OAc)_2$), 4.73(1H,d,J=4.5 Hz) and 4.79(1H,d,J=4.5 Hz)(α,α'-CH-), 6.56(2H,s) and 6.60 (2H,s)(Ar-H). MS(triacetate Xa) *m/z*(%) : 618(M^+, 1.1), 418(3.7), 363(0.8), 351(0.9), 335(2.5), 221(1.5), 205(2.5), 191(5.5), 167(19.4), 159(100), 117 (4.6), 99(10.3), 57(8), 43(54.3).

Compound XI. The malonate ether obtained above was partially reduced to ethyl glycerate-2-(syringaresinol monobenzyl ether) ether with $NaBH_4$ in methanol at 0°C for 1 h with a 43.5% yield, and the ester group was hydrolyzed in 1 N NaOH and dimethylsulfoxide(1 : 5) at room temperature for 2 h. Finally, the benzyl protecting group was removed by the same procedure as described above, giving glyceric acid-2-syringaresinol ether(XI) with about a 72% overall yield from the ethyl glycerate ether. Crystallization was unsuccessful, but the compound was chromatographically pure and used as a substrate without any purification. ^{1}H-NMR(dimethylated derivative XIa)(200 MHz, $CDCl_3$) δ(ppm) : 3.09(2H,m,β,β'-CH-), 3.84, 3.85 and 3.87(18H,three s,Ar-OCH_3 and -$COOCH_3$), 3.75(1H,dd,J=12, 3 Hz,-$C\underline{H}_2$OH;another proton overlapped with OCH_3 signals), 3.94(2H,br dd,J=9.1, 3.8 Hz) and 4.31(2H,br dd,J=9.1, 6.7 Hz)(γ,γ'-CH_2-), 4.54(1H,dd,J=9.1, 3.1 Hz,Ar-O$C\underline{H}$-), 4.74(1H,d,J=4.4 Hz) and

4.75(1H,d,J=4.3 Hz)(α,α'-CH-), 6.57(2H,s) and 6.58(2H,s)(Ar-H). (acetate of XIa, XIb) : 2.06(3H,s,-OCOCH$_3$), 3.10(2H,m,β,β'-CH-), 3.815, 3.822, 3.84 and 3.87(18H,four s,Ar-OCH$_3$ and -COOCH$_3$), 3.93(2H,br dd,J=9.2, 3.8 Hz) and 4.30(2H,br dd,J=9.2, 5.2 Hz)(γ,γ'-CH$_2$-), 4.46(2H,d,J=4.8 Hz, -CH$_2$OAc), 4.72-4.80(3H,m,Ar-OCH- and α,α'-CH-), 6.54(2H,s) and 6.57(2H,s)(Ar-H). IR(XIa) ν_{max}^{KBr} cm^{-1} : 1760(-COOCH$_3$). MS(XIa) *m/z*(%) : 534(M^+, 71.6), 504(6), 432(48.9), 283(5.7), 249(9.2), 235(13.5), 224(11.3), 219(11.3), 207(41.8), 195(46.1), 191(31.9), 181(100), 167(34.3), 161(13.5), 153(17). (XIb) : 576(M^+, 2.4), 516(1.6), 432(5.2), 207(3.1), 195(4), 181(10), 167(3.2), 145(100), 55(14.3), 43(37.2).

Oxidation of Syringaresinol Monobenzyl Ether with Peroxidase/H_2O_2

Compound Ib(125 mg) was dissolved in a mixture of acetone(18 ml) and distilled water(80 ml). To a stirred solution were added 1.5 mg of horseradish peroxidase(Sigma) in distilled water(5 ml) and 6.68 ml of 0.5% H_2O_2 over a period of 15 min. Stirring was continued for 5 h at room temperature and the reaction mixture was extracted with ethyl acetate. The organic layer was washed with a saturated NaCl solution, dried over Na_2SO_4 and evaporated under reduced pressure. TLC of the residue developed with 3% methanol in methylene chloride gave five spots with R_f values of 0.59(A), 0.53(B), 0.34(C), 0.21(D) and 0.18(E). Preparative TLC(1% methanol in methylene chloride) separated the mixture into two fractions ; higher R_f fraction(A and B) and lower R_f fraction(C, D and E). The higher R_f fraction was further separated by TLC(methylene chloride—*n*-hexane,1 : 1,v/v) to give A(13 mg) and B(21.5 mg) which were identified as 2,6-dimethoxy-*p*-benzoquinone(IV) and 6-oxo-2-(4-benzyloxy-3,5-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane(IIIb), respectively. The lower R_f fraction was also separated by TLC(ethyl acetate—*n*-hexane, 1 : 1, v/v). Both D and E showed a similar UV absorption(λ_{max} : 308 and 307 nm) in ethanol which shifted to λ_{max} 370 nm

in 0.1 N NaOH, and a weak IR band at 1683 cm^{-1} in CH_2Cl_2, suggesting the presence of an α-carbonyl group conjugated with *p*-hydroxy aromatic ring : the mass spectra of both D and E showed the molecular ion peak at *m/z* 524 and the base ion peak at *m/z* 91 with other ion peaks at *m/z* 506, 488, 476, 415, 235, 217, 181, 167, 153 and 123. The NMR spectrum(90 MHz) was very complicated and the signals were not assignable. However, both D and E gave a diacetate which was identified as the diacetate of 3-hydroxymethyl-2-(4-benzyloxy-3,5-dimethoxyphenyl)-4-(4-hydroxy-3,5-dimethoxybenzoyl)tetrahydrofuran(IIb-B). Compound C was found to be an artefact.

Compound IIb-Ba(diacetate of IIb-B). ^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 1.62(3H,s,γ'-$OCOCH_3$), 2.32(3H,s,Ar-$OCOCH_3$), 2.71 - 3.09(1H,m,β'-CH-), 3.85 (6H,s) and 3.87(6H,s)(Ar-OCH_3), 4.13(2H,d,J=7.5 Hz, γ'-CH_2-), 4.22 - 4.44(3H, m,β-CH- and γ-CH_2-), 4.77(1H,d,J=7 Hz, α'-CH-), 5.00(2H,s,Ar-CH_2-), 6.61(2H, s) and 7.20(2H,s)(Ar-H), 7.22 - 7.56(5H,m,Ar-H). IR ν_{max}^{KBr} cm^{-1} : 1680(α-C=O), 1750 and 1770(acetyl-C=O). MS *m/z* : 608(M^+), 458, 457, 415, 245, 223, 209, 181, 167, 163, 149, 91.

Compound IIIb. ^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 2.99 - 3.58(2H,m,β,β'-CH-), 3.84(6H,s,Ar-OCH_3), 4.08 - 4.43(4H,m,γ,γ'-CH_2-), 4.58(1H,d,J=6 Hz, α'-CH-), 4.96(2H,s,Ar-CH_2-), 6.47(2H,s,Ar-H), 7.10 - 7.48(5H,m,Ar-H). UV $\lambda_{max}^{Dioxane}$ nm(log ε) : 265.6(3.07), 269.4(3.08). IR ν_{max}^{KBr} cm^{-1} : 1765(α-C=O). MS *m/z* 370(M^+), 279, 251, 236, 91. Mp. 173°C.

Compound IV. ^{1}H-NMR(90 MHz, $CDCl_3$) δ(ppm) : 3.81(6H,s,-OCH_3), 5.86 (2H,s,-CH-). UV $\lambda_{max}^{Dioxane}$ nm(log ε) : 284.3(3.78), 370(2.44). IR ν_{max}^{KBr} cm^{-1} : 1710, 1650, 1630, 1600, 1115. MS *m/z* : 168(M^+), 140, 138, 125, 112, 97, 69.

Two spots(D and E) observed on a TLC plate could be ascribed to the ring-chain tautomerism between hemiketal(IIb-A) and keto-alcohol(IIb-B), which results in the complicated NMR signals and the similar mass spectra

of isolated fractions(D and E). Therefore, D and E were presumed to be a mixture of IIb-A and IIb-B.

Oxidation of γ-Lactone III with Peroxidase/H_2O_2

Compound III(3 mg) in acetone(0.5 ml) was added to a stirred solution of 0.2 mg of peroxidase in 6 ml of distilled water, and then 145 µl of 0.5 % H_2O_2 was added to the mixture. The mixture was stirred at room temperature and a 0.5 ml sample was taken for UV measurement. Spectra were taken after acidification and dilution. After 3.5 h incubation, the reaction mixture was extracted with ethyl acetate. The concentrated extract was analyzed by GC-MS : steel column(1 m) 1.5% SE 30 on Chromosorb W AW, temp. 110°C - 220°C(10°C/min), carrier He 30 ml/min.

Two compounds were identified by mass spectral comparison with the authentic samples as 2,6-dimethoxy-*p*-benzoquinone(IV) and 2,6-dioxo-3,7-dioxabicyclo[3,3,0]octane(VI).

Authentic sample VI was prepared by nitric acid oxidation of dibromosyringaresinol dimethyl ether[26]. MS *m/z*(%) : 142(M^+, 2.7), 112(0.5), 98 (3.1), 97(1.9), 85(7.5), 84(10.4), 82(4.4), 70(11.1), 69(100), 68(10.2), 55(54.2), 54(28.1), 53(15), 41(73.7), 39(47.4).

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CHAPTER III

DEGRADATION OF *d,l*-SYRINGARESINOL AND BIPHENYL COMPOUNDS BY *PHANEROCHAETE CHRYSOSPORIUM*

INTRODUCTION

In this chapter, degradation of *d,l*-syringaresinol and its derivatives by a white-rot fungus *Phanerochaete chrysosporium* (= *Sporotrichum pulverulentum*) was described and their degradation pathways were proposed.

The culture conditions affecting the ligninolytic activity of the fungus have been extensively investigated by Kirk and his co-workers[1-4]. *P. chrysosporium* is only one fungus for which nutritional and environmental controls on lignin degradation were elucidated[5]. In this fungus ligninolytic activity is optimum in low nitrogen, in a high oxygen atmosphere, and in the presence of another carbon source such as glucose or cellulose in surface culture without agitation. These factors have been determined by radioisotopic assay (^{14}C-lignin → $^{14}CO_2$)[6]. This assay can establish the ligninolytic activity (complete degradation of lignin to CO_2) of fungi, but can not distinguish the individual reactions involved in the lignin degradation. Therefore, studies on the chemistry of lignin degradation is also necessary to elucidate the degradation mechanism. For this purpose, studies with model compounds were made with this white-rotter.

Degradation studies with various model compounds have recently been conducted with ligninolytic cultures of *P. chrysosporium* by several workers[7]. In addition, low molecular weight products formed during wood decay by the fungus have been intensively examined by GC-MS[8]. Decayed polymeric lignin

has also been investigated mainly by ^{13}C-NMR spectroscopy[9] which is useful for characterization of the degradation of heterogeneous compound like lignin[10].

Furthermore, degradation of phenolic biphenyl model compounds by the fungus was tested using two dehydrodimers derived from pinoresinol monoethyl ether and guaiacylglycerol-β-guaiacyl ether.

RESULTS

Degradation of resinols by P. chrysosporium

d,l-Syringaresinol(I) and *d,l*-pinoresinol(X) as phenolic models for β-β' linked units in lignin were preliminarily examined in ligninolytic cultures of *P. chrysosporium*. In both cases, yellow to brown color was developed in the cultures. Compound I was readily transformed into several compounds as judged by TLC, whereas compound X was slowly converted to non-extractable yellow material, indicating its polymerization. The slow

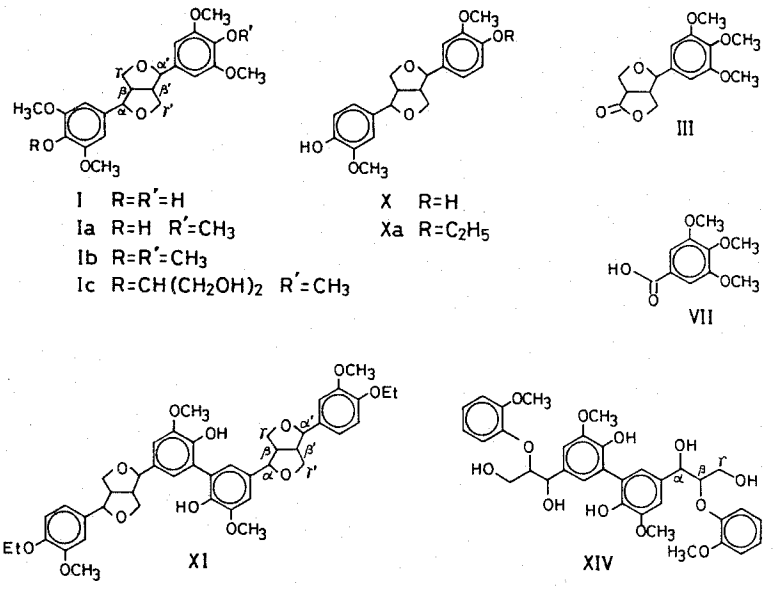

Fig. 1. Structures of compounds tested

reaction of X was partly due to its low solubility in the aqueous medium.

In contrast, *d,l*-syringaresinol dimethyl ether(Ib), non-phenolic model for this type of linkage, was hardly metabolized by *P. chrysosporium*, judging from the UV and TLC analyses of the culture extracts. The ethyl acetate extracts after 6 days incubation were examined for the presence of C_6-C_1 compounds such as 3,4,5-trimethoxybenzoic acid and corresponding aldehyde and alcohol, because C_α-C_β cleavage has been found to be a major degradative reaction for non-phenolic model compounds[7] and lignin[9]. However, none of them were obtained from the extracts. Compounds seen on TLC plate could not be characterized because of their small quantities. This indicated that the propanoid side chain of the non-phenolic resinol is stable in ligninolytic cultures of *P. chrysosporium*.

Hence, *d,l*-syringaresinol monomethyl ether(Ia) and *d,l*-pinoresinol monoethyl ether(Xa) were tested to simplify the products and elucidate the degradation sequence of phenolic resinol by *P. chrysosporium*. Since the fungus is known to synthesize *de novo* veratryl(3,4-dimethoxybenzyl) alcohol[11,12], ethyl ether of X, Xa, was used.

Degradation of syringaresinol monomethyl ether(Ia)

Compound Ia(100 mg) was converted to a number of compounds after 48 h of incubation with *P. chrysosporium*. Compounds A(< 1 mg), B(< 1 mg), C(10.4 mg), D(trace), E(< 1 mg) and F(2.4 mg) were isolated, after acetylation of the ethyl acetate extracts, by repeated TLC separations. Compounds B, C and D were identified as II', III and IV, respectively by comparison with the authentic samples by TLC, ^{1}H-NMR and MS. Compound A could not be separated in pure form from the substrate(acetate), but easily characterized as a mixture of II and Ia(both acetate) by the ^{1}H-NMR spectrum. Finally, separation was achieved by conversion of II to II' and subsequent TLC as

described in Experimental section. Identity of II' was confirmed by comparison with the authentic sample by TLC, ^{1}H-NMR and MS. Recovery of the substrate was about 2.5%. Formation of II' can be ascribed to the enolization of II and subsequent conversion to the stereoisomer II' between the β and β' positions in the acidic medium ; probably II has *cis* II' has *trans* configuration. Compound E was characterized as triacetate of 3-hydroxymethyl-2-(3,4,5-trimethoxyphenyl)-4-(3-methoxy-4,5-dihydroxybenzoyl)tetrahydrofuran(V) based on its ^{1}H-NMR and mass spectra referring to those of II and ethyl 3-methoxy-4,5-diacetoxybenzoate.

Compound V(triacetate). ^{1}H-NMR($CDCl_3$) δ(ppm) : 1.64(3H,s,γ'-$OCOCH_3$), 2.31(3H,s) and 2.32(3H,s)(Ar-$OCOCH_3$), 2.88(1H,m,β'-CH-), 3.85, 3.89 and 3.91(12H,three s,Ar-OCH_3), 4.12(2H,d,J=6 Hz, γ'-CH_2-), 4.2 - 4.42(3H,m,β-CH- and γ-CH_2-), 4.73(1H,d,J=7.5 Hz, α'-CH-), 6.61(2H,s), 7.31(1H,d,J=1.8 Hz) and 7.50(1H,d,J=1.8 Hz) (Ar-H). MS *m/z*(%) : 560(M^+, 9.2), 502(4.3), 500(2.8), 460(5.4), 418(4.3), 283(4.6), 266(4), 256(9.2), 249(22.7), 222(99.5), 209 (19), 195(35.3), 181(18.7), 167(33.5), 153(8), 43(100).

Compound F was characterized as acetate of 6-hydroxy-2-(3,4,5-trimethoxyphenyl)-3,7-dioxabicyclo[3,3,0]octane(VI) based on its ^{1}H- and ^{13}C-NMR and mass spectra referring to those of related analog[13].

Compound VI(acetate). ^{1}H-NMR($CDCl_3$) δ(ppm) : 2.07(3H,s,α-$OCOCH_3$), 2.94 (1H,m,β'-CH-), 3.17(1H,br dd,J=9, 16.5 Hz,β-CH-), 3.84(3H,s) and 3.88(6H,s) (Ar-OCH_3), 3.66(1H,dd,J=5.8, 9.3 Hz) and 4.48(1H,d,J=9 Hz)(γ-CH_2-), 4.05(1H, dd,J=1.7, 9.3 Hz) and 4.14(1H,dd,J=5.8, 9.3 Hz)(γ'-CH_2-), 4.39(1H,d,J=7.9 Hz, α'-CH-), 6.16(2H,s,α-CH-), 6.57(2H,s,Ar-H). ^{13}C-NMR($CDCl_3$) δ(ppm) : 21.2 (-OCO$\underline{C}H_3$), 52.4 and 52.7(β,β'-C), 56.2 and 60.9(-OCH_3), 71.2 and 71.3(γ,γ'-C), 87.1(α'-C), 102.0(α-C), 102.9, 135.9, 137.7 and 153.5(Ar-C), 170.2 (-O$\underline{C}OCH_3$). MS *m/z* (%) : 338(M^+, 50.6), 278(13.6), 267(13.6), 249(38.8),

219(10.4), 207(11.1), 197(51.2), 196(51.8), 195(44.4), 189(18.5), 181(82), 169(27.8), 82(100), 43(91.3).

These results indicated that the major degradation sequence is Ia → II → III + IV. Therefore, compound III was added to cultures of *P. chrysosporium* for examining further degradation. The compound was insoluble and slowly degraded by the fungus. After 6 days incubation, III was converted to several compounds, although it remained as a main component in the extract. 3,4,5-Trimethoxybenzoic acid(VII) was isolated as a main product by preparative TLC and identified by comparison with the authentic sample by TLC, ^{1}H-NMR and MS. Identification of other compounds was not completed, but they were shown to contain no 3,4,5-trimethoxylated benzene ring in the molecules(NMR). No phenolic compounds were detected in the extract(TLC). Acid VII was found to be reduced to the corresponding aldehyde(VIII) and alcohol(IX) in a separate experiment, and a considerable amount of the alcohol IX was accumulated in the culture. The structures of compounds VIII and IX were confirmed by comparison with the synthetic standards by TLC and ^{1}H-NMR.

Polymerization and degradation of pinoresinol monoethyl ether(Xa)

Addition of compound Xa to the cultures resulted in 20% conversion to its dehydrodimer XI condensed at C_5 position in 48 h incubation, and 37% of substrate was recovered. The biphenyl dimer XI was purified as the diacetate and characterized based on its ^{1}H- and ^{13}C-NMR spectra referring to those of substrate(acetate).

Compound XI(diacetate). ^{1}H-NMR($CDCl_3$) δ(ppm) : 2.09(6H,s,Ar-$OCOCH_3$), 3.1(4H,m,β,β'-CH-), 3.87 and 3.89(12H,two s,Ar-OCH_3), 3.85 - 3.95(4H,m) and 4.2 - 4.3(4H,m)(γ,γ'-CH_2-), 1.46(6H,t,J=7 Hz) and 4.09(4H,q,J=7 Hz)(-OCH_2CH_3), 4.73(2H,d,J=4.7 Hz) and 4.81(2H,d,J=4.1 Hz)(α,α'-CH-), 6.80(2H,d,J=1.8 Hz),

6.82 - 6.9(6H,m) and 7.01(2H,d,J=1.8 Hz)(Ar-H). ^{13}C-NMR($CDCl_3$) δ(ppm) : 14.8(-OCH$_2$$\underline{C}H_3$), 20.5(-OCO$\underline{C}H_3$), 54.1 and 54.3(β,β'-C), 56.0 and 56.1(-OCH$_3$), 64.4(-O$\underline{C}$H$_2$CH$_3$), 71.8 and 71.9(γ,γ'-C), 85.5 and 85.8(α,α'-C), 109.5, 112.5, 118.3, 119.8, 131.3, 133.2, 136.9, 139.5, 147.9, 149.5 and 151.5(Ar-C), 168.7(-O$\underline{C}$OCH$_3$).

Other products obtained from the same extract were more polar compounds and their structures could not be ascertained. It seemed that these unknown compounds were derived from the above-mentioned dehydrodimer and/or related oligomers. Result with Xa coincided with the preliminary observation of X, and showed that, in the phenolic guaiacyl model, condensation probably mediated by the fungal phenol-oxidizing enzyme activity proceeded in the initial reactions.

The fate of this dehydrodimer XI in cultures of *P. chrysosporium* was further examined. The biphenyl compound was rather stable, but slowly degraded to give a compound. The product was isolated by preparative TLC from the ethyl acetate extract after 5 days incubation, and identified as 6-oxo-2-(4-ethoxy-3-methoxyphenyl)-3,7-dioxabicyclo[3,3,0]octane(XII) from its ^{1}H-NMR and mass spectra referring to those of compound III.

Compound XII. ^{1}H-NMR($CDCl_3$) δ(ppm) : 1.46(3H,t,J=7 Hz, -OCH$_2$$\underline{C}H_3$), 3.13 (1H,m,β'-CH-), 3.45(1H,br dt,J=4.0, 8.8 Hz,β-CH-), 3.89(3H,s,Ar-OCH$_3$), 4.11 (2H,q,J=7 Hz, -O$\underline{C}$H$_2$CH$_3$), 4.19(1H,dd,J=9.3, 4.0 Hz) and 4.37(1H,approx. t,J=9 Hz)(γ-CH$_2$-), 4.33(1H,dd,J=9.7, 2.1 Hz) and 4.51(1H,dd,J=9.7, 6.8 Hz)(γ'-CH$_2$-), 4.63(1H,d,J=6.7 Hz, α'-CH-), 6.85 - 6.93(3H,m,Ar-H). MS *m/z*(%) : 278(M^+, 100), 249(12), 247(9), 233(14.1), 186(13), 180(21.7), 165(33.7), 152(57.6), 151 (41.3), 149(19.6), 137(42.4), 135(15.2).

About 75% of substrate was recovered from the same extract. These results indicated that biphenyl XI was mainly degraded via alkyl-aryl C-C bond cleavage, after oxidation of benzylic position(C_α).

Degradation of dehydrodiguaiacylglycerol-β-guaiacyl ether(XIV)

In order to obtain further information on the degradation of biphenyl model compound, another type of biphenyl dimer XIV prepared from guaiacylglycerol-β-guaiacyl ether(XIII) was added to the culture of *P. chrysosporium*. This biphenyl XIV was more actively degraded compared with compound XI. After 5 days incubation, the substrate completely disappeared. Only one product was isolated from the acetylated ethyl acetate extract by preparative TLC, and identified as diacetate of glycerol-2-guaiacyl ether(XV) from its ^{1}H-NMR and mass spectra referring to those of a related analog[14].

Compound XV(diacetate). ^{1}H-NMR($CDCl_3$) δ(ppm) : 2.07(6H,s,-OCOCH_3), 3.84(3H,s,Ar-OCH_3), 4.34(4H,d,J=5 Hz, -OCH($\underline{CH}_2$OAc)$_2$), 4.54(1H,quintet,J=5 Hz, -O$\underline{CH}$(CH_2OAc)$_2$), 6.83 - 7.07(4H,m,Ar-H). MS *m/z*(%) : 228(M^+, 2.3), 159(48.5), 124(25.8), 109(13), 99(6.1), 43(100).

Other compounds did not allow the structural elucidation because of their small quantities. The results indicated that biphenyl XIV was also degraded via alkyl-aryl cleavage, followed by reduction of glyceraldehyde-2-guaiacyl ether as a speculative intermediate to give compound XV[14].

Since compound having glycerol-2-aryl ether structure was isolated as a degradation product, an analogous compound, glycerol-2-(syringaresinol monomethyl ether) ether(Ic), was examined for further degradation. However, compound Ic was found to be stable even after 5 days incubation with *P. chrysosporium*(TLC).

DISCUSSION

The degradation pathway for *d,l*-syringaresinol monomethyl ether(Ia) by *P. chrysosporium* is proposed as shown in Fig. 2, based on the identified

products. The main pathway seems to be Ia → II → III(+ IV) → VII in consid-

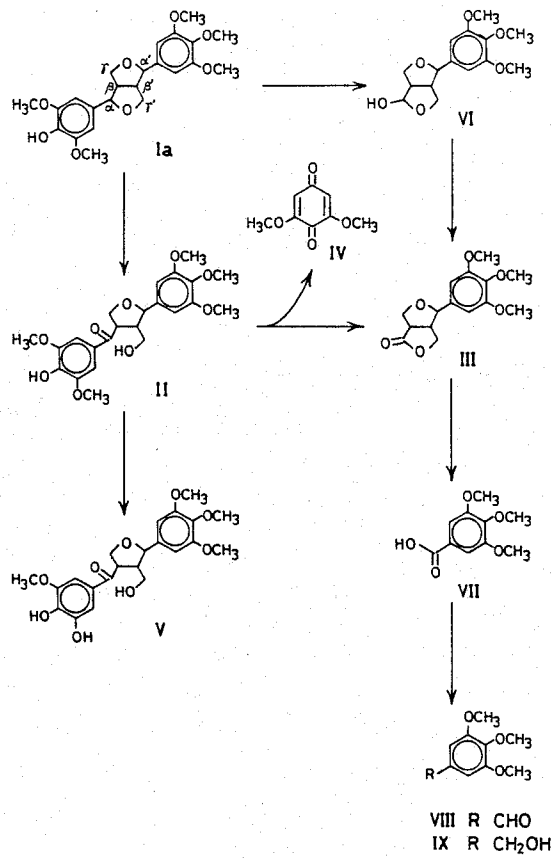

Fig. 2. Proposed degradation pathway for *d,l*-syringaresinol monomethyl ether(Ia) by *Phanerochaete chrysosporium*. The α-oxidized compounds (II and V) can occur in two chemical structures as described in Chapter II : the ring-opened forms(isolated as the corresponding acetates) are illustrated in this figure.

eration of their yields, and suggest the involvement of phenol-oxidizing enzymes. The degradative reaction for the phenolic syringaresinol model described here seems to be common in other white-rot fungi, major lignin degraders, considering the results with a typical white-rotter, *Coriolus versicolor*[13].

P. chrysosporium can degrade compound III to yield VII, indicating that the fungus catalyzes C_α-C_β cleavage of the side chain of III. This type of reaction has been characterized as an important degradative reaction involved in lignin degradation by *P. chrysosporium*[7-9]. Previous studies

(Chapter II) showed that *F. solani* M-13-1 oxidatively degraded I in the same manner, but did not degrade III.

Demethylation of the syringyl nucleus to the catechol structure was also found by the isolation of compound V. However, dealkylation of a non-phenolic nucleus was not found in the present investigation. A catechol structure is considered to be important for the subsequent ring cleavage reaction. Very recently, intra-diol cleavage of a simple catechol to muconic acid by the fungus has been demonstrated using a mutant selected for diminished phenol-oxidizing enzyme activity[15]. The catechol derivative obtained here would undergo ring cleavage reaction in addition to the alkyl-aryl cleavage.

Isolation of compound VI also indicated alkyl-aryl cleavage of Ia. However, the mechanism for the formation of VI from Ia seems different from that giving III. In the degradation of I by *C. versicolor*[13], Katayama isolated an analogous compound which was ascribed to the reduction of a speculative intermediate analogous to III. However, reduction of III to VI was not found in the present investigation. Another possibility for the formation of VI is the reduction of II and subsequent cleavage of the α-hydroxy derivative by phenol-oxidizing enzymes. This seems, not to be the case, because α-hydroxy derivative was not found during incubation of Ia. Compound VI might be oxidized to III, because III was predominant but VI was not present in the extracts after 6 days incubation.

Condensation probably by the phenol-oxidizing enzymes seems to be predominant in the initial reaction of phenolic pinoresinol model Xa. A similar polymerization of X by peroxidase/H_2O_2 and laccase/O_2 has been reported[16]. However, further degradation of the resulting main product, biphenyl dimer XI, led to the alkyl-aryl cleavage to give γ-lactone derivative XII as in the case of syringaresinol having additional methoxyl group instead

of phenyl group at *ortho*(C_5)-position to the phenolic hydroxyl group. Furthermore, as evidenced by the results with another biphenyl compound (XIV) derived from an important β-ether dimer, phenolic biphenyl structure can be degraded via alkyl-aryl cleavage and release more reactive aromatic rings. In both cases(XI and XIV), counterpart products after cleavage reaction were not characterized, but hydroquinone or the corresponding *p*-quinone derivatives were assumed as possible intermediates(Fig. 3.). Apparently, these compounds are metabolized or polymerized faster than XII or XV. Considering the results with vanillic acid metabolism[17], hydroquinone derivatives would undergo ring cleavage reaction.

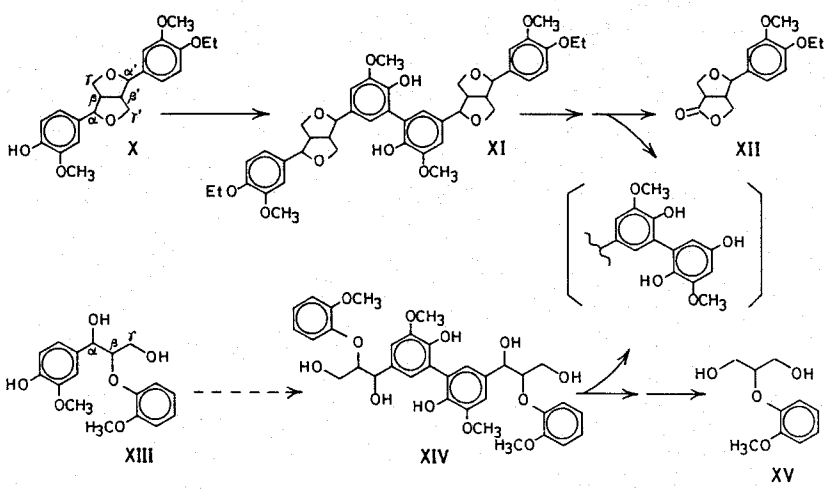

Fig. 3. Alkyl-aryl cleavage of biphenyl model compounds (XI) and (XIV) by *Phanerochaete chrysosporium*.

On the other hand, compound XV has been isolated as a product of compound XIII by *P. chrysosporium*[18]. The accumulation of XV might be ascribed to its relative stability in cultures of the fungus. The stability of glycerol-2-aryl ether structure was supported by a separate experiment with compound Ic.

In contrast to the previous studies with various non-phenolic lignin substructure models[7], *d,l*-syringaresinol dimethyl ether(Ib) was hardly

metabolized by *P. chrysosporium*. C_6-C_1 compounds, which would be released by C_α-C_β cleavage of the side chain, were not detected by TLC and GLC. The resistance of the resinol substructure to microbial attack has been suggested by ^{13}C-NMR spectroscopy using specifically ^{13}C-labeled DHP[19].

It is concluded that resinol substructure is rather stable for degradation by *P. chrysosporium* and active degradation of this type of unit occurs after ether cleavage during attack by the fungus, and phenolic biphenyl structure is degraded via alkyl-aryl cleavage as a major reaction by the fungus.

From the present experiments with biphenyl models, information concerning the aromatic ring cleavage by *P. chrysosporium* was not obtained. This might be explained by rapid metabolism of ring cleavage products[15]. In addition, phenol-oxidizing enzymes would convert the cleavage products highly complicated mixtures.

EXPERIMENTAL

Organism

Phanerochaete chrysosporium Burds. ME-446, obtained from Forest Products Laboratory, U.S. Department of Agriculture, Madison, WI, was maintained at room temperature on 2% malt agar slants, and 1 - 3-week-old slants served as spore inoculum for the experimental cultures.

Media and Culture Conditions

The fungus was grown in the low nitrogen medium[2]. The basal salts medium contained per liter of distilled water : KH_2PO_4, 200 mg ; $MgSO_4 \cdot 7H_2O$, 50 mg ; $CaCl_2$, 10 mg ; mineral solution, 1 ml ; vitamin solution, 0.5 ml[2].

Glucose(10 g) served as carbon source, and L-asparagine(100 mg) and NH_4NO_3 served as nitrogen sources. The medium was buffered with polyacrylic acid (0.01 M, pH 4.5)[20].

The experimental culture was made in a 300 ml Erlenmeyer flask containing 20 ml of the medium and incubated at 39 - 40°C without agitation.

Each substrate(2.5 - 5.0 mg/culture) was added to 5 - 6-day-old cultures as DMF solution(50 - 100 µl), and cultures were flushed with sterile oxygen and incubated in the same condition.

Extraction and Isolation of Degradation Products

The process of the substrate degradation was monitored mainly by TLC of the culture extracts, obtained by extracting one of the replicate cultures with ethyl acetate at intervals. The entire culture was acidified with 1 N HCl to pH 2 and extracted three times with an equal volume of ethyl acetate. Combined extracts were washed with a saturated NaCl solution, dried over Na_2SO_4 and evaporated under reduced pressure at 40°C. The remaining DMF was removed under high vacuum. Precoated silica gel TLC plates(Kieselgel 60 F_{254}, Merck) were used for analytical and preparative TLC. Separation and purification of the products were conducted by preparative TLC using an appropriate mixture of the following solvent systems : methanol - methylene chloride, methylene chloride - *n*-hexane, ethyl acetate - *n*-hexane.

Substrates and Intermediates

Compounds I, Ia, Ib and Ic. *d,l*-Syringaresinol(I) was synthesized as described in Chapter I. Its mono-(Ia) and di-(Ib) methyl ethers were prepared by methylation[CH_3I/NaH/DMF/r.t.] of I and purified by preparative TLC(ethyl acetate : *n*-hexane,4 : 3,v/v). Compound Ic was prepared from glycerol-2-syringaresinol ether(see Chapter II) by methylation with diazo-

methane. ^{1}H-NMR(Ia, $CDCl_3$) δ(ppm) : 3.11(2H,m,β,β'-CH-), 3.84(3H,s), 3.86 (6H,s) and 3.89(6H,s)(Ar-OCH_3), 3.88 - 3.97(2H,m) and 4.45 - 4.57(2H,m)(γ,γ'-CH_2-), 4.74(1H,d,J=4.2 Hz) and 4.76(1H,d,J=4.5 Hz)(α,α'-CH-), 5.52(1H,s,Ar-OH), 6.58(2H,s) and 6.59(2H,s)(Ar-H). (Ib, $CDCl_3$) : 3.11(2H,m,β,β'-CH-), 3.84(6H,s) and 3.87(12H,s)(Ar-OCH_3), 3.93(2H,dd,J=9.2, 3.7 Hz) and 4.31(2H, dd,J=9.2, 6.9 Hz)(γ,γ'-CH_2-), 4.75(2H,d,J=4.4 Hz, α,α'-CH-), 6.57(4H,s,Ar-H). (Ic, $CDCl_3$) : 3.11(2H,m,β,β'-CH-), 3.70 - 3.90(4H,m,-CH_2OH), 3.84(3H,s), 3.88 (6H,s) and 3.90(6H,s)(Ar-OCH_3), 3.95(2H,dd,J=9.3, 3.8 Hz) and 4.28 - 4.38(2H, m)(γ,γ'-CH_2-), 4.05(1H,m,Ar-OC<u>H</u>$(CH_2OH)_2$), 4.76(1H,d,J=4.5 Hz) and 4.77(1H,d, J=4 Hz)(α,α'-CH-), 6.57(2H,s) and 6.61(2H,s)(Ar-H). MS(Ia) *m/z*(%) : 432 (M^+, 100), 249(5.8), 235(10.9), 224(11.6), 207(31.6), 195(31.2), 182(30.7), 181(75.4), 167(37.1), 154(12.3). (Ib) : 446(M^+, 100), 412(19.7), 265(7.4), 249(12.3), 235(14.8), 224(25.4), 219(13.9), 207(76.2), 195(77.9), 181(99.2), 176(26.2), 169(33.6), 153(17.2), 125(15.6). (Ic) : 506(M^+, 36.2), 432(70.5), 235(13.4), 224(14.1), 207(46.3), 195(43), 181(100), 167(42.3), 161(18.8).

Compounds VII, VIII and IX. 3,4,5-Trimethoxybenzoic acid(VII) was commercially available(Tokyo Chemical Industry Co., Ltd.) and recrystallized before use. 3,4,5-Trimethoxybenzaldehyde(VIII) was prepared by methylation [CH_3I/K_2CO_3/DMF/r.t.] of syringaldehyde(Tokyo Chemical Industry Co., Ltd.), and 3,4,5-trimethoxybenzyl alcohol(IX) was prepared by $NaBH_4$ reduction of VIII in methanol at 0°C. ^{1}H-NMR(VIII, $CDCl_3$)δ(ppm) : 3.94(6H,s) and 3.95(3H, s)(Ar-OCH_3), 7.14(2H,s,Ar-H), 9.88(1H,s,-CHO). (IX, $CDCl_3$) : 3.84(3H,s) and 3.87(6H,s)(Ar-OCH_3), 4.63(2H,s,-C<u>H</u>$_2$OH), 6.60(2H,s,Ar-H).

Compounds X and Xa. *d,l*-Pinoresinol(X) was synthesized as previously described[21]. Its monoethyl ether(Xa) was prepared by ethylation[C_2H_5I/NaH/DMF/r.t.] of X monobenzyl ether and subsequent cleavage of the benzyl protecting group[H_2/10% Pd-C/methanol - dioxane(2 : 1, v/v)] and purified by preparative TLC(ethyl acetate : *n*-hexane, 4 : 3, v/v). ^{1}H-NMR(Xa, $CDCl_3$)

δ(ppm) : 1.46(3H,t,J=7 Hz, Ar-$OCH_2\underline{CH_3}$), 3.11(2H,m,β,β'-CH-), 3.89(3H,s) and 3.91(3H,s)(Ar-OCH_3), 3.8 - 3.9(2H,m) and 4.25(2H,br dd,J=9.2, 6.8 Hz)(γ,γ'-CH_2), 4.10(2H,q,J=7 Hz, Ar-$O\underline{CH_2}CH_3$), 4.74(1H,d,J=4.3 Hz) and 4.75(1H,d,J=4.3 Hz)(α,α'-CH-), 5.61(1H,s,Ar-OH), 6.8 - 6.94(6H,m,Ar-H). MS(Xa) *m/z*(%) : 386(M^+, 79.6), 354(8.6), 233(10), 224(9.1), 205(18.8), 191(30.1), 179(45.6), 165(22.1), 152(52.2), 151(100), 137(26.5), 124(19).

Compound XI. This compound was obtained as a degradation product of Xaby *P. chrysosporium* and purified as diacetate(see RESULTS section). Deacetylation of the diacetate by CH_3ONa in 20% methanol/methylene chloride at 0°C gave substrate XI which was purified by preparative TLC(ethyl acetate : *n*-hexane, 1 : 1, v/v). The structure was confirmed by ^1H- and ^{13}C-NMR and used as a substrate.

Compound XIV. This compound was obtained as an oxidation product of guaiacylglycerol-β-guaiacyl ether(XIII, *erythro/threo* mixture) with peroxidase and H_2O_2[22]. The structure was confirmed by ^1H- and ^{13}C-NMR spectroscopy, after purification by repeated TLC separations. ^1H-NMR(acetone-d_6) δ(ppm) : 3.5 - 3.9(4H,m, γ-CH_2-), 3.78, 3.83 and 3.85(12H,three s,Ar-OCH_3), 4.2 - 4.4(2H,m,β-CH-), 4.92(2H,m,α-CH-), 6.75 - 7.25(12H,m,Ar-H). ^{13}C-NMR (acetone-d_6) δ(ppm) : 56.3 and 56.4(Ar-OCH_3), 61.9 and 62.0(γ-C), 74.0(α-C), 86.6 and 88.4(β-C), 110.2 - 151.9(21 signals, Ar-C).

Peroxidative Degradation of d,l-Syringaresinol Monomethyl Ether(Ia)

Compounds II, III and IV as references for degradation products of Ia were prepared by oxidation of Ia with peroxidase and H_2O_2 as described in Chapter II. Compound II was isomerized on a silica gel TLC plate as follows. Compound II(diacetate, 21.5 mg) was spread on a TLC plate and kept at room temperature for 2 weeks. The band was then eluted from the plate. Purification of the mixture gave II diacetate(< 1 mg) and a compound(18.5

mg) which was characterized as stereoisomer(II') of II.

Compound II(diacetate), ^{1}H-NMR($CDCl_3$) δ(ppm) : 1.64(3H,s,γ'-$OCOCH_3$), 2.35(3H,s,Ar-$OCOCH_3$), 2.91(1H,m,β'-CH-), 3.84(3H,s), 3.88(6H,s) and 3.89(6H,s)(Ar-OCH_3), 4.12(2H,d,J=7.4 Hz, γ'-CH_2-), 4.25 - 4.45(3H,m,β-CH- and γ-CH_2-), 4.76(1H,d,J=7.3 Hz, α'-CH-), 6.63(2H,s) and 7.22(2H,s)(Ar-H). ^{13}C-NMR($CDCl_3$) δ(ppm) : 20.2 and 20.4(-$OCO\underline{C}H_3$), 47.1 and 50.6(β,β'-C), 56.2, 56.4 and 60.8(-OCH_3), 70.5 and 76.4(γ,γ'-C), 83.6(α'-C), 102.8, 104.9, 133.3, 135.3, 136.2, 137.6, 152.4 and 153.4(Ar-C), 168.0 and 170.4(-$O\underline{C}OCH_3$), 197.9 (α-C). IR ν_{max}^{KBr} cm^{-1} : 1680(α-C=O), 1745 and 1770(acetyl-C=O). MS *m/z*(%): 532(M^+, 9.1), 490(1.7), 472(2.3), 266(4.1), 249(19.7), 235(4.1), 222(100), 209(15.7), 195(20.3), 181(42.3), 167(4.1), 153(5.4), 55(9.8), 43(36.8).

Compound II'(diacetate). ^{1}H-NMR($CDCl_3$) δ(ppm) : 1.95(3H,s,γ'-$OCOCH_3$), 2.37(3H,s,Ar-$OCOCH_3$), 3.09(1H,m,β'-CH-), 3.85(3H,s), 3.90(6H,s) and 3.91(6H,s)(Ar-OCH_3), 4.04(1H,m,β-CH-), 4.15 - 4.38(4H,m,γ,γ'-CH_2-), 4.62(1H,d,J=8.7 Hz, α'-CH-), 6.67(2H,s) and 7.22(2H,s)(Ar-H). ^{13}C-NMR($CDCl_3$) δ(ppm) : 20.4 and 20.7(-$OCO\underline{C}H_3$), 49.7 and 50.4(β,β'-C), 56.2, 56.4 and 60.8(-OCH_3), 63.1 and 70.9(γ,γ'-C), 84.2(α'-C), 103.5, 105.2, 133.3, 134.3, 135.4, 137.8, 152.5 and 153.4(Ar-C), 168.1 and 170.8(-$O\underline{C}OCH_3$), 197.1(α-C). IR ν_{max}^{KBr} cm^{-1}: 1680(α-C=O), 1745 and 1770(acetyl-C=O). MS *m/z*(%) : 532(M^+, 29.3), 490 (11.4), 266(18), 249(34.1), 235(9), 222(99.4), 209(18), 196(21.6), 195 (29.3), 181(88), 167(7.8), 153(11.4), 55(10.8), 43(100).

Compound III. ^{1}H-NMR($CDCl_3$) δ(ppm) : 3.13(1H,m, β'-CH-), 3.46(1H,br dt, J=3.8, 8.9 Hz,β-CH-), 3.84(3H,s) and 3.88(6H,s)(Ar-OCH_3), 4.22(1H,dd,J=3.8, 9.3 Hz) and 4.40(1H,dd,J=8.9, 9.3 Hz)(γ-CH_2-), 4.36(1H,dd,J=2.2, 9.8 Hz) and 4.53(1H,dd,J=6.7, 9.8 Hz)(γ'-CH_2-), 4.62(1H,d,J=6.9 Hz, α'-CH-), 6.56(2H,s, Ar-H). ^{13}C-NMR($CDCl_3$) δ(ppm) : 46.0 and 48.5(β,β'-C), 56.2 and 60.9(-OCH_3), 69.8 and 70.2(γ,γ'-C), 86.2(α'-C), 102.9, 134.5, 138.0 and 153.6(Ar-C), 178.0(α-C). IR ν_{max}^{KBr} cm^{-1} : 1770(α-C=O). MS *m/z*(%) : 294(M^+, 100), 279

(10.4), 263(9.8), 251(5.8), 224(5.2), 197(23,4), 196(28.6), 195(23.4), 181 (80.5), 169(22.7).

Analytical Instruments

A Hitachi 200-20 double beam spectrometer and a Hitachi 260-30 infrared spectrometer were used for UV and IR spectra, respectively. NMR spectra were taken with a Varian XL-200 NMR spectrometer at 200 MHz for ^{1}H-NMR and at 50 MHz for ^{13}C-NMR using TMS as an internal standard. Mass spectra were measured with a Shimadzu-LKB 9000 gas chromatograph - mass spectrometer (70 eV).

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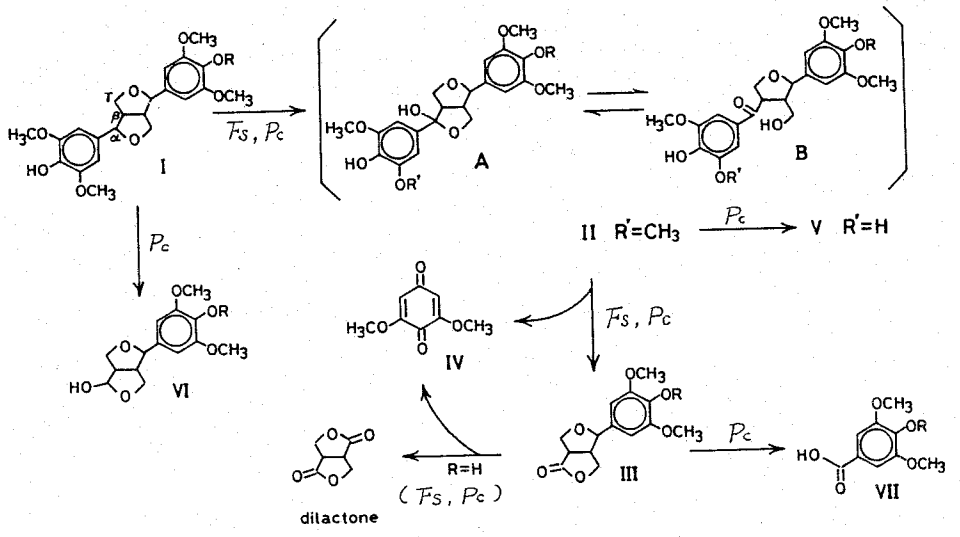
CONCLUSIONS

In Chapter I, the syntheses of *d,l*-syringaresinol(SR) and guaiacylglycerol-β-syringaresinol ether(GSRE) were described. Sinapyl alcohol(SA), a precursor of lignin, was synthesized by $LiAlH_4$ reduction of methyl 4-*O*-(α-ethoxy)ethyl sinapate and subsequent deprotection of ethoxy ethyl ether. By this procedure, the alcohol was obtained with a high yield. SR was prepared by dehydrogenation of SA with peroxidase and hydrogen peroxide system in 66.5% overall yield from methyl 4-*O*-(α-ethoxy)ethyl sinapate. GSRE was synthesized from SR according to the β-ether synthetic method via β-hydroxy ester as a key intermediate in 23% overall yield.

In Chapter II, degradation pathways for SR and GSRE by a soil fungus *Fusarium solani* M-13-1 were discussed. SR was degraded via alkyl-aryl(C_α-C_1) bond cleavage by the fungal phenol-oxidizing enzymes(mainly laccase), but its dimethyl ether(SRDM) was not degraded by the fungus. SR moiety in GSRE was similarly attacked by the fungus, and the arylglycerol moiety was also degraded by the alkyl-aryl cleavage yielding 2-aryl ethers of glcerol or glyceric acid. In addition, β-ether linkage was splitted by unknown mechanism in cultures of *F. solani*. When syringylglycerol-β-syringaresinol ether was used as a substrate, considerable amounts of α-keto derivatives, common structure found in decayed wood lignins, were found. The use of higher oligolignols was expected to provide more valuable information which had not been obtained with dilignols.

In Chapter III, degradation pathways for SR and *d,l*-pinoresinol(PR) by a white-rot fungus *Phanerochaete chrysosporium* Burds.(ME-446) were

discussed. The main degradative reaction of SR by the fungus was essentially the same as was found in *F. solani*. However, some different and important degradative reactions were found to occur in ligninolytic cultures of *P. chrysosporium*. 3-*O*-demethylation of syringyl nucleus to give a catechol structure and C_α-C_β side chain cleavage of the γ-lactone intermediate were characterized from the degradation products. The former reaction was presumed to be important for subsequent ring cleavage reaction. In addition, another type of alkyl-aryl cleavage was found, but the mechanism remained unclear.



Degradative reactions of syringaresinol substructure by *Fusarium solani* M-13-1(Fs) and *Phanerochaete chrysosporium* Burds.(Pc); R=H or Alkyl

PR was first condensed mainly at C_5 position to give its biphenyl dimer. However, the biphenyl dimer was also degraded via alkyl-aryl cleavage by the fungus. This was further demonstrated with another type of biphenyl derived from β-ether dimer, guaiacylglycerol-β-guaiacyl ether. Therefore, phenolic guaiacyl models could finally be degraded in the same manner as in the case with syringyl models. The aromatic rings thus released were presumed to be highly reactive and undergo ring cleavage reaction in cultures

of *P. chrysosporium*. However, information concerning the ring cleavage reaction could not be obtained.

The present studies indicate that *d,l*-syringaresinol(I, R=H) could be finally decomposed to *p*-quinone IV and *d,l*-dilactone by phenol-oxidizing enzyme activity of these fungi. Further studies with polymeric lignin will be required to confirm the relevance of the reaction described here to lignin biodegradation.

Both fungi could not metabolize non-phenolic resinol model SRDM. This seems to indicate that the non-phenolic β-β' substructure is considerably stable for the fungi, although it was recently found by the present author that a white-rot fungi *Coriolus versicolor* metabolized SRDM slowly.

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